Vol. XXIII, No. 6 1929

THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

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CAMBRIDGE UNIVERSITY PRESS
LONDON: Fetter Lane, E.C. 4
also
H. K. LEWIS & Co., Ltd., 136, Gower Street, London, W.C. 1
CHICAGO: The University of Chicago Press
(Agent for the United States)
BOMBAY, CALCUTTA, MADRAS: Macmillan and Co., Ltd.
TOKYO: Maruzen Company, Ltd.

1929

Price Twenty-five Shillings net

PRINTED IN GREAT BRITAIN

The *Biochemical Journal* is conducted by the Biochemical Society and is published by the Cambridge University Press.

This Society has been instituted for the purpose of facilitating intercourse between those biologists and chemists who are interested in the investigation of problems common to both, such as the chemical problems connected with Agriculture, Brewing, Animal and Vegetable Physiology and Pathology, &c. Persons interested in Biochemistry are eligible for election.

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CXXIV. SOME LIVER OILS YIELDING A STRONG COLOUR REACTION WITH ANTIMONY TRICHLORIDE[1].

By SIGNE and SIGVAL SCHMIDT-NIELSEN.

From the Laboratory of Applied Organic Chemistry, Trondhjem.

(*Received September 17th, 1929.*)

During recent years the authors [1928, 1; 1929, 1] have been preparing oils from different raw materials for the purpose of investigating the practical value of the antimony trichloride test for the detection and quantitative estimation of vitamin A [Carr and Price, 1926]. It has been shown that fat from certain marine animals yields a particularly strong blue colour. Our investigations on the biological effect of these products are not yet finished, but the results found are of such interest as to justify a preliminary communication.

Reaction of fish-liver oils with antimony trichloride.

As is known, the antimony trichloride test is carried out by adding 2 cc. of a saturated solution of antimony trichloride in chloroform to 0·2 cc. chloroform containing 0·04 cc. oil. The colour intensity for ordinary cod-liver oil measured with a Rosenheim-Schuster colorimeter amounts to six Lovibond blue units, the time for the reading being 1 minute. Many different kinds of fishes, including both cartilaginous and bony ones, give values of the same order of magnitude. Thus 0·04 cc. of oil obtained by boiling the liver of the porbeagle (*Lamna cornubica* Gmel.)—caught January, 1927—gave six Lovibond blue units. As usual, large quantities of liver oil were obtained by extraction of the remainder with ether, but this fraction showed to our great surprise a very strong colour reaction, viz. several hundred Lovibond blue units for 0·04 cc. (see below). This striking difference shows that the colour-producing substance must be differently bound to the liver cells from the fat obtainable by melting.

Not all liver oils, however, behave in this manner. Thus cod-liver treated with boiling water gave a liver oil that in some cases showed eleven Lovibond blue units for 0·04 cc. Upon extraction, however, the remainder yielded a colour intensity which lay only a few units higher than that of the directly melted part. It appears that in this case colour-producing substances are also

[1] Communicated at the 18th meeting of the Scandinavian naturalists in Copenhagen, August 28th, 1929.

present in the liver which do not directly follow the melted fat, but the quantity is much higher for porbeagle-liver than for cod-liver.

We have also examined the liver oil from other fishes and particularly from those which do not yield any oil by direct heating of the liver, but only by extraction with a solvent. It then appeared that the liver from the halibut, salmon, red perch, and mackerel upon extraction also gave a strong colour reaction with antimony trichloride. This was true also for whale-liver, which—like that of all mammals—does not yield any liver oil upon heating with water (the fat content did not exceed 4 g./100 g.).

The determination of the colour intensity for these liver oils gives rise to some difficulty. Readings above 30 blue units cannot be effected with the tintometer, and the optimum is between 5 and 15. This necessitates dilution of the samples, the colour, however, is not proportional to the degree of dilution, but somewhat stronger.

Thus, the following values were found for the liver oil from the mackerel:

cc. oil in 0·2 cc. chloroform (2 cc. of the reagent)	Colour intensity	Calculated for 0·04 cc.
0·002	14·7	294
0·001	11·0	440
0·0004	5·8	580
0·0002	3·4	680

At lower concentrations the values for 0·04 cc. lie still higher. Under these circumstances it is impossible to use one reading for the colour obtainable. We have therefore plotted the colour intensities against dilution and read off from this curve the quantity of liver oil that would correspond to six Lovibond blue units, *i.e.* the average value for a good cod-liver oil. From this value is calculated the colour intensity for 0·04 cc. of the oil, all readings being made after exactly one minute. The values found (see Table I) represent minimum values, since the colour in many cases is perceptibly fading after 1 minute. Parallel readings have been made for all dilutions, and these show good concordance. The light conditions are of some influence, and the values obtained at different hours of the day are therefore not always the same.

Table I. *Showing the Lovibond blue units for* 0·04 *cc. oil.*

Liver oil from the halibut (*Hippoglossus hippoglossus*), sample FE	700
Liver oil from the salmon (*Salmo salar*), sample FD	200
Liver oil from the salmon (*Salmo salar*), sample FN	250
Liver oil from the mackerel (*Scomber scombrus* Lin.), sample FL	570
Liver oil from the red perch (*Sebastes marinus*), sample FG ...	70
Peritoneal fat from red perch (*Sebastes marinus*), sample FH ...	13
Liver oil from porbeagle (*Lamna cornubica*), sample HDA melted	1·6
Liver oil from porbeagle (*Lamna cornubica*), sample HDB extracted	270
Liver oil from porbeagle (*Lamna cornubica*), sample II *a*, melted	6
Liver oil from porbeagle (*Lamna cornubica*), sample II *b*, extracted	420
Liver oil from whale (*Balaenoptera borealis*), sample FM	150

The values for yellow and red on the Lovibond scale were also determined together with the blue colour, but these results are less trustworthy, so we do not give them here. Attention may nevertheless be called to the fact that

the intensity in yellow and red changes to a marked degree both absolutely and relatively. Some liver oils give a pure blue colour which slowly changes into violet and red, whereas others give a reddish violet tint at once. The tendency towards fading of the colour is also very different for the different materials, and this is also true of the final colour obtained after the blue tint has disappeared. This phenomenon is linked up with the content of unsaponifiable matter, as previously pointed out by the authors. This clearly shows that several substances—not only a single one—react with the antimony trichloride under the conditions prevailing and that the colour observed is produced by all these reactions.

Vitamin A content of extracted liver oils.

The investigations on the content of vitamin A in the samples mentioned here have not as yet been brought to a conclusion, but for all that, the data obtained at the present stage are remarkable. Although not particularly potent the melted liver oil from porbeagle (HDA) proved to be sufficient for a satisfactory growth, when it was applied in daily doses of 1–2 mg. The

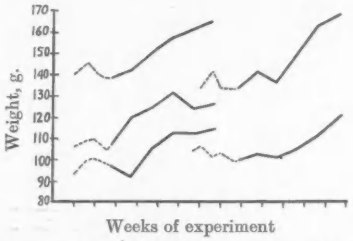

Fig. 1. Weight curves of rats, 0·04 mg. halibut-liver oil a day, corresponding to 25,000 units of vitamin A.

same results were also obtained with the extracted liver oil, and we were disposed to conclude from this result that the content of vitamin A in both samples corresponded to 1000–500 units. This proved, however, to be quite erroneous. The extracted liver oil gives still more excellent growth, if it is applied in great dilutions, *e.g.* in doses of 0·1 mg. a day, and the content of vitamin A is therefore in reality not 1000, but about 10,000 units. The liver oil from the halibut is active in doses of 0·04 mg. a day, as is shown in Fig. 1, which gives the curves of growth for five rats fed on this dose. This consequently means that the liver oil from halibut contains not less than 25,000 units of vitamin A.

Similar results have been obtained with liver oil from salmon (FD) which gave normal growth in daily doses of 0·1 mg., *i.e.* the oil contains in reality 10,000 units instead of 600, as we originally found [1928, 2].

Agduhr [1926] published some three years ago very interesting investigations on the poisonous effect of too heavy doses of cod-liver oil. His results exactly confirm experiments which were made in this laboratory five years

ago, but which have not yet been published in detail [see Schmidt-Nielsen, 1929].

Whether the antimony trichloride reaction can be used for the assay of vitamin A in liver oils yielding a strong blue colour will not be discussed here, since the biological analyses are not yet finished.

Effect of puberty on vitamin A requirements of rats.

Mention may here be made of the importance of puberty for vitamin research. Four of the curves drawn in Fig. 1 have a normal slope in the first part of the test period, but then a more or less pronounced break occurs. This break is coincident with the beginning of puberty, *i.e.* the animals are 3 months old. At this age the animals need vitamin A in larger quantities, and since they are also more sensitive a decline or a stoppage of growth will occur if the doses given are not rather high. If the dose is only sufficient for producing a small, constant growth, then it may happen that the animals die during this period, although the doses and the entire procedure remain unchanged. Normal growth of the animals will, however, reappear after 1 to 3 or 4 weeks, if the original dose has been sufficient.

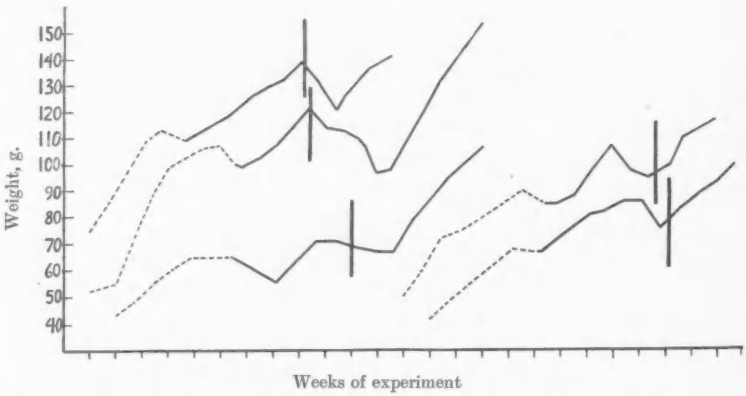

Fig. 2. Influence of puberty. The age of 3 months is marked by a broad, vertical line. The rats from the beginning of the test period had an adequate supply of vitamin A.

This phenomenon is clearly demonstrated in Fig. 2. The age of 3 months is marked by a vertical line, and it will be seen that the curves all bend down at about this point. Then follows a period of normal increase again, and the second part of the curves seems to have the original slope. At first we believed that the periods of decrease were due to a deterioration of the preparations, but the phenomenon appeared also when fresh mixtures were used [Schmidt-Nielsen and Schmidt-Neilsen, 1929, 2].

It is clear that false conclusions may be drawn, if the period of examination is too short, and the 3 months' mark should therefore be drawn in all laboratory journals. The investigations should also be continued for a sufficiently long period, eventually until the rats are 5 months old. A survey of the literature—*e.g.* the results published by various authors in this *Journal*—indicates that most of the curves show similar breaks, and that these occur on the whole at the age of 3 months. But, as far as we know, this fact has not previously been explained as a puberty phenomenon.

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CXXV. THE PRODUCTION OF FAT FROM CARBOHYDRATE AND SIMILAR MEDIA BY A SPECIES OF *PENICILLIUM*.

By HAROLD HAYDEN BARBER.

From the Department of Chemistry, University College, Nottingham.

(*Received September 20th, 1929.*)

Whilst it is generally considered that the origin of fats found in the vegetable kingdom is some form of carbohydrate, the mode of formation of the numerous fatty acids from relatively few carbohydrates is still a matter of speculation and the subject of considerable investigation.

The two principal views advanced to explain the occurrence of the various fatty acids in vegetable fats and oils are somewhat opposed, but both assume that the parent substance is glucose either as such or in the form of starch.

E. Fischer appears to have been the first to suggest that glucose or other hexose molecules are condensed, and, after oxidation and reduction, give higher fatty acids directly, thus accounting for the predominance of C_{18} fatty acids in natural fats and oils. From the C_{18} acids the formation in the animal body of palmitic acid and the lower members of the series has been explained by the β-oxidation theory of Knoop [1904], Dakin [1908, 1, 2, 3; 1909, 1, 2] and Embden *et al.* [1906]. This explanation accords with the fact of the almost universal occurrence in natural fats of acids containing an even number of carbon atoms. Later it was suggested by E. F. Armstrong and Allen [1924] that the C_{18} unit of starch rather than three molecules of glucose might be utilised by the plant to give C_{18} acids more directly, a change involving disruption of the oxygen linkage in the starch molecule followed by a direct union of the carbon atoms.

The other view of the mode of formation of vegetable fatty acids, and one which gives a more satisfactory explanation of the presence of the lower acids, is that glucose is broken down into acetaldehyde [Nencki, 1878; Hoppe-Seyler, 1879] and by aldol condensation the fatty acid skeleton is built up step by step by the addition of carbon atoms two at a time [Magnus Levy, 1902]. The experiments of Raper [1907] confirm this view so far as the lower fatty acids are concerned. A similar suggestion has been advanced by Smedley [1911] and by Smedley and Lubrzynska [1913], according to whom acetaldehyde—derived from pyruvic acid—condenses with a second molecule of pyruvic acid to give an unsaturated α-keto-acid, which on losing carbon dioxide gives

crotonaldehyde. The crotonaldehyde by similar condensations with pyruvic acid gives higher unsaturated aldehydes which are finally oxidised to acids of varying length of carbon chain and of varying degree of unsaturation.

A third view more recently advanced by Smedley-MacLean and Hoffert [1926] appears to reconcile the above two hypotheses by the suggestion, based on experiments with yeast, that acetaldehyde is first converted into hexose and the latter directly condensed to form higher fatty acids without passing through a fatty aldehyde stage, probably by a direct linking of the hexose molecules in a manner not yet known or suggested.

In the course of an investigation on the products of the growth of a pure culture of a species of *Penicillium* on various common carbohydrate media, the author [1927] considered the possibility of testing the general theory of direct fatty acid synthesis from hexose molecules by the method of cultivating the mould on solutions of individual sugars as the sole source of carbon and comparing the nature of the fatty products in relation to the carbohydrate employed. It follows that if the mechanism of fatty acid synthesis is direct combination of glucose molecules to give C_{18} fatty acids, normal growth on a pentose medium, if fatty acids are produced at all, should not yield C_{18} acids, although possibly C_{15}, C_{10} or C_5 acids might be produced.

The organism in question was found by preliminary experiments to grow on both xylose and arabinose as readily and as freely as on glucose or sucrose. Xylose was selected for the investigation because of the identity of its configuration with that of the α, β and γ carbon atoms of glucose. Mass culture of the mould on this medium yielded fat in amount similar to that produced from glucose or sucrose. The fatty acids present, whichever medium was employed, were found to consist of a mixture of palmitic, stearic, oleic and α- and β-linoleic acids—linolenic acid being definitely absent. Free acids were present to some extent and resembled those combined as glycerides. Small amounts of sterols were isolated, which will be the subject of a separate investigation. The saturated acids, as indicated by their mean molecular weights and melting points, were apparently a mixture of palmitic and stearic acids.

From time to time several workers have claimed the isolation of daturic acid from various natural sources, and in view of the fact that Dieterle, Diester and Thimann [1927] report the presence of daturic acid in the oil of *Secale cornutum* it was thought desirable that the saturated acids should be further examined. Since the usual methods of differentiation are difficult to apply in any case and are obviously out of the question where the amount of material is so small, recourse was had to X-ray analysis, and through the kindness of Mr Piper of Bristol University, who examined three samples of acids by this means, it was found (*a*) that the combined saturated fatty acids from glucose, xylose and glycerol were all identical, and (*b*) that all these mixtures showed distinct evidence of the presence not of a C_{15} acid but of one containing 19 carbon atoms, together with stearic acid.

Examination of the spent or partially spent medium yielded very little except unchanged carbohydrate and traces of a yellow solid obtained by prolonged extraction of the filtered medium with ether. This substance consisted of a neutral ester and an acid of mean molecular weight (by titration) of 160 to 200; both acid and ester were insoluble in light petroleum but soluble in ether and readily soluble in alcohol. Oxalic acid and volatile acids were entirely absent.

In the case of glycerol growth proceeded much more slowly than on either the pentose or hexose.

Experimental.

The particular species of *Penicillium* used in this investigation was isolated by the author from various moulds found growing on maize-starch pastes used in the finishing of cotton textile fabrics.

A single spore culture was obtained in the usual manner by means of successive dilutions in sterile water and plating on to a 5 % sucrose—2 % agar medium containing Czapek's solution, the same medium in tubes being used for preserving the stock cultures. During the course of the work the stock culture was plated out from time to time in order to ascertain and ensure its continued purity.

Pure cultures of the *Penicillium* were grown in round flat-bottomed litre flasks each containing 500 cc. of liquid. The medium, containing 3, 5, 10 or 20 % of the carbohydrate with the addition of Czapek's solution was sterilised in moist steam at 95–100° on three successive days and the flasks kept for at least 10 days before inoculation. The xylose used was Difco brand and had M.P. 149–149·5°.

The temperature generally employed for cultivation was 21° since it was found that at temperatures only a few degrees lower, *e.g.* 17°, growth was much retarded in every case.

After several weeks, when a thick surface mat had been formed, this was removed, washed in water and partially dried between several layers of filter paper and finally *in vacuo* over sulphuric acid. Meanwhile, the filtered culture medium, which was of a deep orange-red colour, was extracted six times with ether and the ethereal solution washed twice with water. This ether extract gave on evaporation in a stream of dry carbon dioxide a brown solid substance insoluble in light petroleum (40–50°). The total yield was about 0·1–0·8 per litre of culture medium. The substance when treated with dilute sodium hydroxide solution and ether gave a neutral ester and an acid. The ester, which was insoluble in light petroleum, on saponification yielded acids also insoluble in petroleum and resembling the free acid originally present. These acids gave dark-coloured blue-black solutions in dilute sodium carbonate solution from which the acids were precipitated as a yellow solid on adding dilute sulphuric acid. By extraction with ether they were obtained as a dark brown fatty solid, melting above 100°. Determination of mean molecular weight by titration

with $N/10$ sodium hydroxide solution in neutral alcohol with phenolphthalein gave the following values:

0·165 g. acids from 5 % glucose medium. Mean molecular weight 172.

0·032 g. acids from 10 % glucose medium. Mean molecular weight 160.

0·447 g. acids from 5, 10 and 20 % sucrose medium. Mean molecular weight 198.

On oxidation with moderately concentrated nitric acid the principal product was oxalic acid.

The yields of surface mat etc. are summarised in Table I.

Table I. *Yield of surface growth, etc.*

No. of flasks each containing 500 cc. medium	Concentration of sugar (%)	Temp. ° C.	Duration of growth (days)	Total ether extract from medium g.	Dry surface mat	
					g.	g. per flask
Glucose:						
4	5	17	98	0·09	9·0	2·25
4	5	17	34	0·03	9·0	2·25
8	10	17	34	0·14	15·8	2·00
4	5	21	21	0·36	15·0	3·75
5	5	21	21	0·36	15·0	3·75
8	5	21	35	0·59	35·0	4·38
Sucrose:						
7	5	21	28	—	26·0	3·70
4	5	21	34	0·26	13·0	3·25
4	10	21	34	0·27	10·0	2·50
4	20	21	34	0·33	12·0	3·00
8	5	21	35	—	30·0	3·75
Glycerol:						
8	10	21	80	—	21·0	2·63
3	10	21	87	1·17	9·0	3·00
8	10	21	96	2·40	22·0	2·75
8	10	21	201	—	50·0	6·25
Xylose:						
4	3	21	33	0·16	13·0	3·25
6	3	21	60	0·32	21·0	3·50

The dry surface mat, finely powdered and extracted with dry ether in a Soxhlet apparatus yielded a pale yellow viscous oil. This was treated with dilute sodium hydroxide solution and ether to remove free acids as soaps, the latter were then extracted with ether after acidifying their aqueous solutions with dilute sulphuric acid. The free acids were pale yellow solids and on treatment with light petroleum yielded a very small proportion of darker insoluble acids presumably hydroxy-acids. The free fatty acids taken up by the light petroleum were examined as regards their melting points, mean molecular weights and iodine values in the manner described below for the examination of the combined fatty acids.

The neutral fat after removal of free acids was quantitatively saponified with $N/2$ alcoholic potassium hydroxide solution and after removal of the alcohol in a stream of carbon dioxide on the water-bath the solution was made alkaline with dilute sodium hydroxide solution and the sterols etc. removed by extraction three times with ether. The extracted material appeared to be sparingly soluble in ether and separated as a fine white powder at the interface

of the two liquids. The total ether extract was finally washed with water till free from alkali. The ethereal solution and suspension were then run into a weighed beaker, evaporated and the weight of the residue taken as "crude sterols." From the aqueous soap solution the combined acids were obtained by acidifying with dilute sulphuric acid and extracting with ether. The ethereal solution after being washed was evaporated and the residue of combined acids was taken up in light petroleum leaving traces of insoluble hydroxy-acids. The combined fatty acids after determination of their melting point were titrated with $N/10$ sodium hydroxide solution and phenolphthalein in the presence of at least 50 % by volume of neutral alcohol and their mean molecular weight thus obtained. The iodine value (Wijs) was then determined on a portion of the acids and another portion was brominated in ethereal solution at 0°. No insoluble hexabromides were ever obtained, but on the removal of the ether and the addition of light petroleum (40–50°), boiling and filtering, the tetrabromide of α-linoleic acid was deposited from the cooled filtrate and after recrystallisation from alcohol melted at 114–115°. The fraction of the brominated acids which was soluble in cold light petroleum was weighed, titrated with $N/10$ sodium hydroxide solution and found to have a molecular weight indicative of the presence of soluble tetrabromides of β-linoleic acid.

These results are summarised in Table II.

Table II. *Fat from surface mat grown at 21°.*

Concentration of sugar (%)	Weight of mat extracted (g.)	Free fatty acids: Weight (g.)	Free fatty acids: M.P.	Free fatty acids: Mean molecular weight	Free fatty acids: Iodine value (Wijs)	Neutral fat and sterols: Weight (g.)	Neutral fat and sterols: Sapn. value	Combined fatty acids: M.P.	Combined fatty acids: Mean molecular weight	Combined fatty acids: Iodine value (Wijs)	Combined fatty acids: α-Linoleic acid (%)	Crude sterols % in neutral fat
Glucose:												
5	30	0·33	33	285	96	2·02	186	34	280	90	9·4	—
10	20	0·14	35	295	91	1·08	188	34	288	93	12·0	—
5	31	0·83	37·5	288	81	4·92	191	37	286	88	—	2·5
Sucrose:												
5	26	0·18	36	282	70	2·03	195	36	291	80	—	13·0
5	33	0·15	37	301	84	1·85	186	37	281	90	13·0	—
10	10	0·05	37	298	78	0·54	201	37	282	78	7·0	13·6
Glycerol:												
10	30	0·22	35	306	78	3·21	185	35	284	86	8·4	4·5
10	22	0·09	37	—	88	1·53	176	37–39	283	101	14·5	9·9
10	50	0·33	35–36	281*	—	5·45	184	32	289	98	7·5	7·9
Xylose:												
3	13	0·22	36	288	—	0·86	183	35	279	91	10·0	9·7
		0·12	37	279	—	0·77	189	38	280	—	—	5·8
3	21	0·31	36	292	76	1·15	166	35–36	296	80	—	22·1

* Solid fraction only.

The combined fatty acids were further examined by quantitative separation into solid and liquid fractions by the lead salt-alcohol method of Twitchell [1921]. The melting points and mean molecular weights of the saturated acids thus obtained were then determined. The results, summarised in Table III, suggest the presence of palmitic and stearic acids, but, whatever may be the ultimate composition of the mixture, there is no evidence of the formation of lower acids when xylose is employed.

Table III. *Saturated fatty acids.*

Source		M.P.	Mean molecular weight	% of saturated acids in fatty acids
Glucose	Combined acids	55	264	27
Sucrose	Combined acids	54	271	29
Glycerol	Combined acids	55	275	16
Glycerol	Free acids	55	281	22
Xylose	Combined acids	54	293	21

The X-ray measurements obtained by Mr Piper, indicating the presence of a C_{19} acid are as follows:

	How mounted	Spacings
Acids from glucose	Pressed	Two spacings show in third order not accurately measurable.
	Melted	One spacing 40·45 Å units.
glycerol	Pressed	Two spacings in third order one too faint to measure, the other 43·9 Å units.
	Melted	One spacing 41·15 Å units.
		An equimolar mixture of C_{18} and C_{19} acids gives 43·5 pressed, 41·6 melted, suggesting that the lines photographed are due to these two acids with a high proportion of C_{18}.
xylose	Pressed	Two spacings in third order too diffuse to measure.
	Melted	One spacing 43·5 Å units. (43·7 for equimolar C_{19} and C_{20} melted.)

Discussion.

The remarkable constancy of the analytical values obtained for the fats produced by the organism from both pentose and hexose would appear to indicate that the mechanism of fatty acid synthesis is more complex than the mere condensation of monosaccharides.

Since the final products are identical it seems safe to assume that there is a common intermediate in each case and that the hexose and pentose are both broken down with approximately equal readiness to this substance before the process of actual synthesis begins, the actual synthesis then following a common course.

A similar relationship between pentoses and hexoses in biochemical synthesis by fungi is that shown by the recent work of Challenger, Klein and Walker [1929] on the production of kojic acid by *Aspergillus oryzae*. Whilst it was previously known that kojic acid was produced from glucose by various species of *Aspergillus* it was stated that none was obtained from pentoses. These workers show, however, that *A. oryzae* grows readily on xylose or arabinose,

giving, not pyromeconic acid, as might be expected, but kojic acid. This non-formation of pyromeconic acid, they point out, is remarkable, especially as the formation of kojic acid involves a breakdown of the pentose molecule and the synthesis of a 6-carbon compound.

What the intermediate compound may be, whether it is the same in fatty acid synthesis as in the formation of kojic acid, and whether it is a 3-carbon compound as suggested by the work of Peterson, Fred and Schmidt [1924] or the acetaldehyde of alcoholic fermentation is a problem still awaiting solution.

Summary.

(1) The species of *Penicillium* under investigation grows equally well on solutions of glucose, sucrose or xylose but less freely on glycerol.

(2) The same fat or mixture of fats is produced in every case.

(3) This fat contains palmitic, stearic, oleic and α- and β-linoleic acids both free and as glycerides together with some sterols.

The author wishes to express his thanks to the Chemical Society for a grant towards the cost of material, to the Council of University College, Nottingham, for a maintenance grant during a part of the period, to Mr Piper for his kindness in carrying out the X-ray examination, and to Professor F. S. Kipping, F.R.S., for his kind interest throughout this work.

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CXXVI. THE PREPARATION OF THE UNCONJUGATED ACIDS OF OX-BILE.

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(*Received October 8th, 1929.*)

It has been known for some time that deoxycholic acid is able to bring about lysis of Pneumococci, and that its lytic action is about ten times as great as that of cholic acid. Nevertheless there has not hitherto been available a simple and reliable method of preparing deoxycholic acid which is sufficiently pure for use in bacteriological work. Of the principal methods of preparing deoxycholic acid, those of Pregl [1903] and Pregl and Buchtala [1911] are expensive and difficult to carry out. The method of Langheld [1908] is said by Wieland and Sorge [1916] to be impracticable. The method of Schryver [1912] is reliable but very lengthy, and for that reason is unsuitable for the regular production of deoxycholic acid. The method of Mair [1917] is simple, but the product, though sometimes satisfactory, in my experience is often so contaminated with higher fatty acid that it is quite useless for bacteriological work. Also the method has been found to be unworkable with some samples of ox-bile. Of the methods of preparing cholic acid, the simplest and most recent is that of Wieland and Weil [1912], but it has the disadvantage that only cholic acid is obtained, and that different procedures are necessary for summer bile and for winter bile.

This research was commenced with the object of finding a simple and reliable method of preparing deoxycholic acid suitable for bacteriological work, and in this paper two methods of preparation are described, one of which gives maximum yields of both deoxycholic acid and cholic acid, and the other is suitable for use when deoxycholic acid only is required and the yield of cholic acid is of secondary importance.

According to Wieland and Sorge [1916], choleic acid is a compound of deoxycholic acid with higher fatty acids. If this is crystallised from glacial acetic acid, the higher fatty acids are displaced, and a deoxycholic-acetic acid compound is obtained. This when pure melts at 145°. Pure deoxycholic acid, uncombined with fatty acid, melts at 173°. In this investigation the final substance arrived at was the deoxycholic-acetic acid compound, sometimes also called acetic-choleic acid.

Method I.

This method gives a maximum yield of both deoxycholic acid and cholic acid, and works successfully with ox-bile at any season of the year. Up to 10 litres of bile may be conveniently worked up at a time.

The principle of the method is first to convert the conjugated bile acids into the unconjugated form by hydrolysis of the bile with sodium hydroxide. The acids of the bile are then treated as a 10 % solution of their sodium salts by fractional precipitation with magnesium chloride solution. Magnesium deoxycholate is much less soluble in water than magnesium cholate, and also the magnesium salts of the higher fatty acids are less soluble than magnesium deoxycholate. Consequently the first precipitate, which is usually rejected, consists principally of useless magnesium soaps, which, if not removed, would interfere with the subsequent crystallisation of the second precipitate from 60 % acetic acid. The second precipitate, which is almost free from pigment, consists of the magnesium salts of most of the deoxycholic acid, and of some cholic acid. The bulk of the cholic acid remains in solution. The magnesium deoxycholate which has been precipitated is freed from admixture with cholic acid, and the free acid at the same time regenerated by crystallisation from 60 % acetic acid. The product is freed from fatty acid by converting it into the xylene-deoxycholic acid compound and this is reconverted into acetic-choleic acid by crystallisation from glacial acetic acid. Cholic acid is recovered from two sources, the solution which is left after the precipitation of the magnesium deoxycholate, and the 60 % acetic acid filtrate. From the former it is precipitated by acidification, decolorised with charcoal, and any deoxycholic acid still present precipitated by treating it as a 2 % solution of its sodium salts with magnesium chloride after Schryver's method. The magnesium cholate solution is acidified, and the precipitated cholic acid rubbed up with cold alcohol and filtered off. The cholic acid which is thrown out of solution on pouring the 60 % acetic acid filtrate into water is similarly rubbed up with cold alcohol in order to remove impurities which render very difficult the crystallisation of the crude material from hot alcohol in the ordinary way. This treatment also removes pigment and any remaining deoxycholic acid more efficiently than ordinary crystallisation from alcohol, the latter because the deoxycholic acid, which is present in an uncombined form or combined with acetic acid, is very easily soluble in cold alcohol and is easily removed by it, but in hot alcoholic solution it would combine with higher fatty acid to form a compound which is only moderately soluble in alcohol. After one treatment with cold alcohol, the cholic acid is recrystallised from hot absolute alcohol in the ordinary way.

Hydrolysis of the bile. For the purpose of hydrolysis, ox-bile obtained fresh from the slaughter-house is heated almost to boiling in a covered iron vessel for 20 hours with sodium hydroxide, 60 g. of this substance, dissolved in 100 cc. of water, being employed for each litre of bile. Any fluid lost by evaporation

is then replaced by the addition of water, and hydrochloric acid is added to the hot solution until a drop of it just fails to turn phenolphthalein pink. About 180 cc. of strong hydrochloric acid is required to neutralise each 60 g. of added soda, and if the solution is sufficiently hot and is carefully stirred, it can be added without precipitating any of the bile acids. After cooling, the neutralised solution is made definitely acid by the addition of hydrochloric acid with stirring to precipitate the bile acids. The precipitate is allowed to settle for a few hours; with different samples of bile it varies from a thick oil to a pasty mass, which becomes brittle on standing.

Separation of the greater part of the deoxycholic acid as a magnesium salt. The supernatant fluid is decanted from the pasty mass of hydrolysed bile acids, which is kneaded to free it from excess of water and mineral acid, and added to a boiling 10 % solution of sodium hydroxide, of which about 60 cc. is used for each litre of bile which is being worked up. The mixture is heated and stirred, more soda being added if necessary, until the solid material has dissolved. The excess of soda is then neutralised to phenolphthalein by adding dilute acetic acid to the vigorously stirred and boiling-hot solution. The solution is then diluted to 600 cc. for each litre of bile, which gives a solution of the solid material of the bile of 6–10 % strength. To this is added one-fiftieth of its volume of 40 % magnesium chloride solution, the mixture stirred, and heated for 1 hour at 90–100°. When the mixture is cold it is filtered, the supernatant liquor being decanted into the filter as completely as possible before the precipitate is thrown on to it. The precipitate (precipitate 1 *a*) is washed on the filter with a little cold water, and if it is in the slightest degree pigmented and greasy should be rejected, as in that case it is largely composed of uncrystallisable fatty substances. Sometimes, however, it is a white perfectly granular substance, and in that case it may be further treated in a way to be described. To the filtrate is added one-tenth of its volume of 40 % magnesium chloride solution, and the mixture heated, with occasional stirring, for 1 hour at 90–100°. A very heavy precipitate is produced, the solution setting almost to a paste. When it is cold, this is filtered off on the pump, filtration being quick and easy, and the precipitate is washed on the filter with several quantities of cold water, and pressed with a spatula until a perfectly white and only slightly moist substance is left on the filter (precipitate 1 *b*). The further treatment of the filtrate (filtrate 1 *b*) from which cholic acid and a further small amount of deoxycholic acid are obtained, is described below.

Crystallisation of the precipitated magnesium salts from 60 % *acetic acid.* Precipitate 1 *b* is dried at 100–110° until it can be crumbled to a powder. This is dissolved in four times its weight of boiling 60 % acetic acid and left for 2 days to crystallise out, crystallisation being aided if the mixture is stirred after standing a day. The crystals are then filtered off and washed on the filter with small quantities of cold 60 % acetic acid till free from pigment (precipitate 1 *c*).

Precipitate 1 *a* usually consists of fatty material which, if it were not

removed, would prevent the second precipitate of magnesium salts from crystallising from acetic acid. Occasionally, however, when less of these fatty substances is present in the bile, it is a white, perfectly granular, rather bulky precipitate, and in this case it should be treated in the same way as precipitate 1 *b*. As, however, it contains a certain amount of inorganic matter, its solution in 60 % acetic acid must be filtered whilst boiling-hot. Any material crystallising from the filtered solution may then be added to precipitate 1 *c*.

Removal of fatty acid by boiling with xylene. The material crystallised from the 60 % acetic acid is dried for 1 hour at 100°, powdered, and boiled vigorously under reflux in a conical flask for about 3 hours with eight times its weight of xylene. At the end of this operation the mixture is allowed to become perfectly cold, and the xylene-deoxycholic acid compound filtered off on the pump, washed with a little cold xylene and well drained (precipitate 1 *d*). The filtrate contains only some fatty acid and a trace of bile acid and pigment, and the xylene is easily recovered from it by distillation.

Conversion of the xylene-deoxycholic acid compound into deoxycholic acid by crystallisation from glacial acetic acid. The xylene-deoxycholic acid compound (precipitate 1 *d*) is dried for 1 hour at 100–110°, dissolved in four times its weight of glacial acetic acid, the solution boiled for half a minute and left to crystallise. After standing 24 hours the crystals (precipitate 1 *e*) are filtered off, washed on the filter with a very little cold glacial acetic acid, and well drained. They are colourless, and consist of nearly pure deoxycholic acid combined with acetic acid (acetic-choleic acid). The M.P. is usually 137–141° or higher, and the material is pure enough for bacteriological purposes. It may be further purified by recrystallisation from four times its weight of glacial acetic acid.

From the mother liquors of any further crystallisations from glacial acetic acid, a further crystallisation of less pure deoxycholic acid, M.P. 130–135°, can be obtained by evaporation to about one-third of their volume.

Treatment of filtrates.

The filtrate 1 *b from the precipitation of the magnesium salts* contains the greater part of the cholic acid and pigment of the bile, and a certain amount of deoxycholic acid. It is acidified with dilute hydrochloric acid and stirred, the precipitate allowed to settle, collected in one mass, kneaded to free it from water and mineral acid, and added to a hot 10 % solution of sodium hydroxide, about 30–40 cc. of a 10 % solution being required for each litre of bile. The solution is warmed and stirred, more soda being added if necessary, until the solid material has all been converted into sodium salts and dissolved. To the very hot solution dilute acetic acid is added, with careful stirring, until it is only weakly alkaline, and then 20–25 g. of charcoal for each litre of bile is stirred into the solution. The mixture is now evaporated to dryness and finally dried in the air-oven at 100–110°. The dry mass is then powdered, and extracted with methylated spirit for 12–15 hours in a Soxhlet apparatus to remove the sodium salts of the bile acids.

The methylated spirit extract is evaporated to dryness, and a 2 % aqueous solution of the residue is made. To this solution, which is only lightly coloured, one-tenth of its volume of a 40 % solution of magnesium chloride is added, the mixture stirred, and heated for 1 hour at 90–100°. A precipitate of magnesium deoxycholate is produced, and is filtered off when the mixture is cold. This precipitate is treated in the same way as the precipitate 1 *b* which was obtained at an earlier stage of the preparation, and both deoxycholic acid and cholic acid can be obtained from it.

The filtrate is acidified with acetic acid, stirred, allowed to stand overnight and the precipitate collected, kneaded to free it from water, and rubbed up in a strong-walled beaker to a thin cream with 2–3 volumes of methylated spirit, sufficient being used to leave a thin layer of clear pigmented fluid above the material when it is allowed to settle. The mixture is left overnight in the ice-chest, and the finely crystalline mass is then filtered off and washed with small quantities of cold methylated spirit, on the filter, until a perfectly white powder is left. This is impure cholic acid, M.P. 185–190°. It is easily purified by two recrystallisations from absolute alcohol.

The filtrate 1 *c from the crystallisation from* 60 % *acetic acid* contains a large amount of cholic acid. It is poured into about ten volumes of water, left overnight, the supernatant liquor decanted off, the precipitated solids kneaded to free them from water, and rubbed up to a cream with methylated spirit in the same way as the precipitate whose treatment is described in the previous paragraph. It is most important that the methylated spirit cream should not be heated or the product will be much less pure. On filtering off the material after standing overnight, and after careful washing on the filter with small quantities of cold methylated spirit, a perfectly white powder is obtained which is impure cholic acid, M.P. 190–195°.

Method II.

This is shorter than method I, but the yield of deoxycholic acid is rather less, and only a small amount of cholic acid is obtained. The method is usually reliable, but occasionally with some samples of bile it may be impossible to obtain a satisfactory crystallisation from acetic acid of the crude product from the hydrolysis. In such cases method I must be used. Up to 10 litres of bile may be conveniently worked up at a time.

The principle of this method is very similar to that of the method of Mair [1917], the separation of the two bile acids depending upon the fact that cholic acid is easily soluble in 60 % acetic acid or glacial acetic acid, whilst deoxycholic acid is comparatively insoluble in these solvents. The deoxycholic acid is freed from admixture with fatty acid by converting it into the xylene-choleic acid compound, and cholic acid is obtained from the second and third acetic acid filtrates, and purified by treatment with alcohol, on the lines described in connection with method I.

Hydrolysis of the bile. This operation is carried out in the same way as in method I, except that the bile acids are precipitated from the neutralised

solution after hydrolysis by adding glacial acetic instead of hydrochloric acid. This avoids the presence of a dangerous amount of mineral acid when the bile acids are subsequently heated with 60 % acetic acid. About 20 cc. of glacial acetic acid are required for each litre of bile, and whilst it is being added the solution is stirred round with a glass rod, on which the precipitated bile acids are collected into a single pasty mass which can be easily removed.

Crystallisation of the crude hydrolysed bile acids from acetic acid of about 60 % *strength.* As much fluid as possible is kneaded out of the precipitate, and it is weighed and dissolved by heating in rather more than an equal weight of glacial acetic acid and left to crystallise out. After standing for 4 days the crystals are filtered off on the pump, freed from pigment by washing on the filter with small quantities of cold 60 % acetic acid, and well drained. The filtrate is rejected.

Crystallisation from glacial acetic acid to remove cholic acid and inorganic impurities. The material crystallised from 60 % acetic acid is dissolved in three parts of glacial acetic acid, and the boiling-hot solution is filtered by suction through a Büchner filter of ample size. To ensure easy filtration the filter funnel should be heated beforehand, and the filter paper moistened with hot glacial acetic acid. After standing for 24 hours, the material which has crystallised out is filtered off by suction, washed with a very little cold glacial acetic acid on the filter, drained, and dried for 1 hour at 110°. The filtrate (2 *b*) is treated as described below. The dried material is further treated by the procedure described in method I, commencing at the stage where the precipitate 1 *c* is boiled with eight times its weight of xylene. The final product is deoxycholic acid, combined with acetic acid, M.P. 141–144°, similar to that obtained by method I. The filtrate (filtrate 2 *b*) is treated in exactly the same way as the filtrate 1 *c* obtained from the crystallisation from 60 % acetic acid in method I. Impure cholic acid, M.P. 190–195°, is obtained, and is finally purified by recrystallisation from absolute alcohol.

Yields.

The yields per litre vary considerably with different samples of bile.

Method I.

Deoxycholic acid	M.P. 141–144°	10–13 g.
Deoxycholic acid[1]	M.P. 130–135°	1–2 g.
Cholic acid[2]	M.P. 185–195°	19–23 g.

Method II.

Deoxycholic acid	M.P. 141–144°	6–8 g.
Deoxycholic acid[1]	M.P. 130–135°	1–2 g.
Cholic acid[2]	M.P. 190–195°	6–8 g.

[1] Second crystallisations from later glacial acetic acid mother liquors.

[2] Weighed before final purification by recrystallisation from absolute alcohol.

Preparation of uncombined deoxycholic acid from acetic-choleic acid.

As the deoxycholic-acetic acid compound produces lysis of Pneumococci perfectly satisfactorily, it is unnecessary to remove the acetic acid from material which is to be used for bacteriological work. It is, however, worth while mentioning two methods of doing this which have proved satisfactory.

1. *By digesting acetic-choleic acid in dilute sodium hydroxide solution.* Pure acetic-choleic acid is dissolved in 100 volumes of $N/10$ sodium hydroxide solution, and the solution heated under reflux for 5 hours on the water-bath. After cooling the solution is acidified, with careful stirring, with the least possible amount of dilute hydrochloric acid. The precipitated deoxycholic acid is filtered off, washed on the filter with water, and dried *in vacuo* at room temperature for 1–2 days, and then for a few hours at 100–110°. Very careful drying is essential in order to remove all trace of hydrochloric acid: M.P. of product 169–172°.

2. *By distilling off the acetic acid in a current of steam.* Pure acetic-choleic acid is finely powdered and poured into five volumes of boiling water in a flask, and a strong current of steam is blown through the mixture for 5 hours. The acetic-choleic acid first changes into a viscous yellow fluid, which, as the acetic acid is removed, should form a powdery suspension, which collects into hard lumps.

After 5 hours the mixture is cooled, the deoxycholic acid filtered off and dried *in vacuo* as before: M.P. of product 169–172°.

The deoxycholic acid thus prepared can be crystallised from alcohol, but the procedure involves considerable loss, owing to the great solubility of the material.

In conclusion I have to thank Prof. H. S. Raper, F.R.S., for his constant interest, encouragement and advice.

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CXXVII. THE COLORIMETRIC DETERMINATION OF RHAMNOSE.

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(*Received September 24th, 1929.*)

The estimation of the methylpentoses, of which rhamnose is the best known, is usually carried out by distilling the material for analysis with 12 % HCl. Methylfurfuraldehyde is formed and can be estimated in the distillate gravimetrically, or by one of the methods based upon its reducing power [Pervier and Gortner, 1923]. These distillation methods require much time and attention and are essentially macro-methods.

The micro-method now to be described is accurate; it is also rapid because large numbers of estimations can be carried out simultaneously. The principle is similar to that of the method already described for the estimation of pentoses [McCance, 1926].

Methylfurfuraldehyde (unlike furfuraldehyde) does not form a coloured compound with aniline or benzidine, both of which have been used for the colorimetric estimation of the simple pentoses such as xylose and arabinose [Youngburg and Pucher, 1924; Hoffman, 1927]. With phloroglucinol, however, methylfurfuraldehyde forms a soluble coloured compound which has been adapted to the estimation of rhamnose.

Solutions and reagents required.

(1) Pure concentrated HCl.
(2) Benzene.
(3) 0·25 % phloroglucinol in 98 % alcohol.
(4) 0·1 % solution of rhamnose (standard).

Procedure.

3 cc. standard, 3 cc. water and 3 cc. concentrated HCl are mixed in a test-tube. An amount of the unknown solution containing about 3 mg. of rhamnose is put in a second test-tube. Water is added to 6 cc. and finally 3 cc. concentrated HCl. The whole is mixed. Unknown and standard are then placed in a water-bath at 100° for 2 hours, each with a cork fitted with a long glass

[1] Working with a part-time grant from the Medical Research Council.

tube as an air condenser. After removal from the water-bath and cooling, 4 cc. of benzene are added both to the unknown and to the standard and the test-tubes are vigorously shaken for at least 3 minutes. They are then allowed to stand for 20 minutes. By spinning between the fingers and by lifting the tube an inch and allowing it to drop back into its place in the rack the benzene layer above the unknown can be readily cleared of black "humic" matter which may appear in it after shaking. 2 cc. of the supernatant benzene of the unknown and standard are then removed with a pipette and placed in clean dry test-tubes. 4 cc. of phloroglucinol solution are added to each and finally, with shaking, 0·65 cc. of concentrated HCl. A deep yellow colour at once appears which continues to develop for about 3 minutes. If the unknown solution is urine, the colour match should be made between 3·5 and 4 minutes after adding the HCl, for something is extracted by the benzene from urine under these conditions which slowly develops a colour with phloroglucinol indistinguishable from that of the methylfurfuraldehyde compound. This does not affect estimations carried out 4 minutes after adding the HCl. The colour of the standard remains unchanged for at least 0·5 to 1 hour so that a large number of separate unknowns may be estimated in succession against one standard, each being matched 3·5–4 minutes after adding the HCl. The reading of the unknown should not be far removed from that of the standard if accuracy is desired.

Scope and limitations of the method.

(1) The following figures illustrate some of the recoveries which have been obtained by this method when known amounts of rhamnose have been added to a urine. (The solutions were made up by one and estimated by a second person.)

Rhamnose added (mg. per 100 cc. urine)	Rhamnose recovered (mg. per 100 cc. urine)
200	200
100	99
67	65
50	54

(2) When hexoses are boiled with HCl, small amounts of hydroxymethylfurfuraldehyde are formed in amounts varying with the hexose. These substances react with phloroglucinol giving a yellow colour similar to that produced by methylfurfuraldehyde. The error introduced into the estimation of rhamnose by the presence of amounts of hexose equal to the rhamnose has been determined and found to be: with fructose 3·0 %; with glucose 1·4 %; with mannose 2·0 %; with galactose 0·35 %.

(3) The presence of other pentoses (arabinose or xylose) interferes with this method as phloroglucinol forms a deep blackish green compound with furfuraldehyde.

SUMMARY.

A colorimetric method of estimating rhamnose is described, based upon the reaction between phloroglucinol and methylfurfuraldehyde. The methylfurfuraldehyde formed by heating rhamnose with hydrochloric acid is separated, for estimation, by extraction with benzene and not by distillation.

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CXXVIII. EFFECT OF PENTOSE INGESTION ON URIC ACID EXCRETION.

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(*Received October 10th, 1929.*)

It has been stated by Thomas and his co-workers [1927, 1, 2] in a series of papers that the ingestion of small amounts of pentoses exerts a decided influence on the excretion of nitrogenous bodies by the kidneys. One of the results claimed was an increased excretion of uric acid commencing some 6 hours after the pentose had been taken by the mouth. Were this correct, it might be of definite therapeutic importance in the treatment of gout, for the pentoses are absolutely non-toxic and compare very favourably in this respect with atophan. Again, as sucrose even in doses of over 200 g. has been found by Mendel and Stehle [1915] to have a negligible effect upon uric acid excretion, the effect of pentoses would be somewhat specific and therefore of theoretical interest. The experiments were repeated therefore upon three healthy adult males in the following way. Each was the subject of three experiments; with xylose, arabinose and rhamnose respectively. 5 g. of the pentose dissolved in about 100 cc. of water were taken by mouth on a fasting stomach. At the same time samples of blood were taken for uric acid estimation and the bladder was emptied. Urine was collected at 15 or 30 minute intervals for 2 hours and thereafter at intervals of 1–2 hours till the end of the 9th hour. Samples of blood were taken for uric acid determinations at various times during the day. Estimations of pentose and uric acid were carried out on the specimens of urine. The subjects were allowed a very light lunch of bread and cheese 4 hours after the pentose had been ingested.

The estimations were made by the following methods.

(1) Uric acid in urine by Folin's method [1925] as set forth by Beaumont and Dodds [1926].

(2) Uric acid in blood by Folin's method [Beaumont and Dodds, 1926] with the added step of precipitating the uric acid as the silver salt. This was deemed a wise precaution in view of the work of Hunter and Eagles [1925] on ergothioneine in blood and the colour this compound is known to give with the uric acid reagent.

(3) Xylose, arabinose, and rhamnose in urine by McCance's methods [1926, 1929].

[1] Working with a part-time grant from the Medical Research Council.

Fig. 1 shows the results of one typical experiment in detail. It will be noted (*a*) that the excretion of pentose is almost over in 6 hours; (*b*) that there is no rise of uric acid excretion 6 hours after taking the pentose; (*c*) that although the uric acid excretion is variable from hour to hour it bears no relation to that of the pentose. The sudden fall coincident with the peak of pentose excretion is fortuitous and not shown in our other experiments. Hourly variations in the excretion of uric acid are known to occur, although no satisfactory explanation has been advanced to account for them. They are apparently not caused by fluctuations in the level of uric acid in the blood. On averaging a series of experiments these spontaneous fluctuations disappear and the following figures have been obtained by taking the average of the nine separate experiments which have been carried out.

Time after pentose ingestion (hrs)	Average rate of uric acid excretion per hour (mg.)
1–3	19·44
3–6	19·6
6–9	18·8

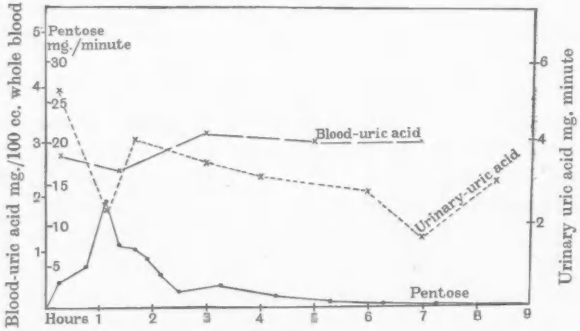

Fig. 1.

Uric acid and pentose curves:

Each point is plotted in the centre of the period during which that particular specimen of urine was secreted. It indicates in mg. the average amount of uric acid or arabinose excreted per minute over that period of time.

Blood-uric acid curve:

Each point indicates in mg. per 100 cc. of whole blood the uric acid present at the time the specimen was taken.

The average uric acid excretion remains very constant for these three persons and certainly does not appear to be affected by the ingestion of pentose. There is no evidence here of any regular daily fluctuation [Mendel and Stehle, 1915].

Carbohydrate such as glucose in doses of upwards of 100 g. administered by mouth raises the uric acid excretion of fasting persons who have a high blood-uric acid and a low uric acid excretion [Lennox, 1925]. It is probable that pentose would do the same, but we have no evidence on this point.

SUMMARY.

No evidence has been obtained that the ingestion of pentose has any influence upon the excretion of uric acid.

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CXXIX. NOTE ON THE ACETYL DERIVATIVES OF THYROXINE.

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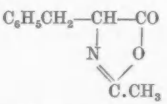
(*Received October 25th, 1929.*)

In the course of some experiments in another laboratory [Gaddum, 1929] regarding the physiological activity of various derivatives of thyroxine, it became desirable to include the "acetylthyroxine" of Kendall and Osterberg [1919] in the series of compounds under investigation. Further, the precise nature of the substance designated "acetylthyroxine" by Kendall is left open to doubt by the description given in his paper. It was, therefore, thought to be worth while to study the compound further from the chemical as well as from the physiological side. The physiological results are contained in a paper by Gaddum [1929]; the present note deals with the chemical investigation which has revealed an unexpected and interesting point.

The first experiment consisted in carrying through a preparation precisely as described by Kendall and Osterberg [1919]; an alcoholic solution of the sodium salt of thyroxine was treated with excess of acetic anhydride, the product was dissolved in alcohol and the solution poured into a large volume of boiling dilute sulphuric acid. The crystalline compound obtained in this way gave no nitrous acid reaction and was insoluble in boiling dilute sodium carbonate; it was evident, therefore, that both the phenolic and carboxylic groups were blocked, *i.e.* that the substance was, in reality, the ester of diacetylthyroxine; this conclusion was confirmed by analysis, and we then proceeded to try the reaction on another amino-acid to see whether the phenomenon of simultaneous acetylation and esterification could be observed in an independent instance. On subjecting phenylalanine to similar treatment we were able in fact to demonstrate the conversion of part of the amino-acid into the ester of its acetyl derivative. It appears, therefore, that the reaction is a general one, and there can be little doubt but that it proceeds through the stage of the azlactone. Bergmann, Stern and Witte [1926] have demonstrated the readiness with which phenylalanine, on treatment with acetic anhydride, is converted into the azlactone



and compounds of this type are known to pass with ease, on treatment with

water or alcohol, to the corresponding acid or ester respectively. In the case under consideration, alcohol being present in excess from the beginning, the existence of the azlactone would naturally be only transient. In any case it is sufficiently obvious that this treatment of thyroxine does lead to simultaneous complete acetylation and esterification, and that the "acetyl sulphate" described by Kendall and Osterberg [1919] (which was, however, not analysed for sulphur) was in fact the diacetylthyroxine ester; the figures given by Kendall for the iodine content of this compound agree well with those required by the ester in question.

In his paper Kendall describes a second method of "purification" of the acetyl derivative, in which the reaction product obtained by treatment of thyroxine with acetic anhydride in alcoholic solution is dissolved in alcohol to which has been added excess of strong sodium hydroxide, the alcohol being subsequently removed by distillation under diminished pressure, and the product isolated as the sodium salt. This product is, needless to say, entirely different from that obtained by the first method of preparation outlined above. It is an acid, and gives an intense nitrous acid reaction, and we were able without difficulty to demonstrate its identity with *N*-acetylthyroxine prepared by an entirely different method.

In his book on thyroxine Kendall [1929] devotes a paragraph to the discussion of the acetyl derivative; in this he seems to have become aware of the complete acetylation accompanying treatment with acetic anhydride and alcohol and he describes yet another method of working up the reaction product, which involves the use of sodium hydroxide, and again leads to *N*-acetylthyroxine; he makes, however, no mention of the esterification which we have shown to take place.

Experimental.

Diacetylthyroxine ethyl ester. According to the instructions of Kendall and Osterberg [1919], 100 mg. of thyroxine were dissolved in 20 cc. of alcohol containing 100 mg. of sodium hydroxide; the solution was treated with 2 cc. of acetic anhydride and allowed to stand for 30 minutes; 5 cc. of water and 5 cc. of 50 % sulphuric acid were added, and the alcohol was removed by distillation under diminished pressure, the temperature of the bath not being allowed to rise above 40°; the white solid which separated was filtered off, washed with water and dissolved in 15 cc. of alcohol; the filtered solution was then poured into 200 cc. of boiling water containing 5 cc. of 50 % sulphuric acid. After standing overnight the product was filtered off and dried. It was recrystallised by dilution with water of its solution in a mixture of four parts alcohol and one part methyl ethyl ketone, and was thus obtained in clusters of colourless needles, which melted at 216–217° after sintering at 213°.

The compound failed to give the nitrous acid reaction, and was insoluble in sodium carbonate solution even at the boiling-point. On analysis it gave figures in agreement with those required by diacetylthyroxine ethyl ester.

Analysis. 64·0 mg. gave 0·974 mg. N (micro-Kjeldahl).
62·4 mg. gave 65·4 mg. AgI.

	N	I
Found	1·5 %	57·1 %
Calculated for $C_{21}H_{19}O_6NI_4$	1·6	56·6

Action of acetic anhydride on phenylalanine in alcoholic solution. *dl*-Phenylalanine (1·65 g.) was dissolved in alcohol (30 cc.) in which 0·23 g. of sodium had been dissolved; 5 cc. of acetic anhydride were added and the mixture was allowed to stand for 30 minutes. At the end of this time the alcohol was removed by distillation under diminished pressure. On addition of water to the residue a part passed into solution; the insoluble oil remaining was taken up in ether, and the ethereal solution was washed with dilute sodium carbonate, dried and evaporated. The residue consisted of a light brown oil which, on keeping overnight, crystallised. It was distilled and had B.P. 155–160° at 2 mm.; the distillate solidified at once, and, on recrystallisation from a mixture of ether and light petroleum, yielded fine colourless needles melting at 67°.

Cherbuliez and Plattner [1929] have recently described acetylphenylalanine ethyl ester, resulting from the action of acetic anhydride on phenylalanine ester, as having B.P. 151–157° at 2 mm. and M.P. 68°. We ourselves prepared some of the ester by esterification of acetylphenylalanine; the product had M.P. 68°, and the melting-point of a mixture of this preparation with the product of the action of acetic anhydride and alcohol on phenylalanine melted at 67–68°.

N-*Acetylthyroxine methyl ester.* A solution of 0·8 g. of thyroxine methyl ester [compare Ashley and Harington, 1928] in 16 cc. of redistilled anisole was cooled to 0° and treated gradually with 0·04 g. of acetyl chloride dissolved in 2 cc. of anisole; after standing for some time at 0°, the precipitate of thyroxine methyl ester hydrochloride was filtered off and the filtrate evaporated under diminished pressure, the temperature not being allowed to rise above 50°. The residue was dissolved in warm benzene; on spontaneous evaporation of the solvent, the ester crystallised out; on recrystallisation from anisole it formed colourless plates of a somewhat irregular shape, melting at 208–209° with decomposition after sintering at 205°.

Analysis. 1·02 mg. required 5·8 cc. *N*/200 thiosulphate [Kendall, 1914].

	I
Found	60·7 %
Calculated for $C_{18}H_{15}O_5NI_4$	61·0

N-*Acetylthyroxine.* The above ester was dissolved in 50 % alcoholic *N* sodium hydroxide (two equivalents); after keeping for 30 minutes at the ordinary temperature the solution was neutralised with the calculated amount of *N* hydrochloric acid. The precipitated acid was at first oily but rapidly hardened; for purification it was dissolved in boiling dilute sodium carbonate, the sparingly soluble sodium salt, which separated on cooling, being filtered

off and decomposed with dilute hydrochloric acid. The free acid was crystallised by pouring its concentrated solution in hot glacial acetic acid into a large volume of boiling water. The substance gave an intense nitrous acid reaction. On heating it sintered at 205° and melted with decomposition at 210–215°.

Analysis. 50·7 mg. gave 0·816 mg. N (micro-Kjeldahl).

0·74 mg. required 4·28 cc. $N/200$ thiosulphate [Kendall, 1914].

	N	I
Found	1·6 %	61·7 %
Calculated for $C_{17}H_{13}O_5NI_4$	1·7	62·0

The above compound was identical in all respects with products obtained (*a*) by the second "method of purification" described by Kendall and Osterberg in their paper [1919], and (*b*) by the method more recently described by Kendall in his book [1929].

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CXXX. CARBOHYDRATES OF CRAB NERVE.

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(*Received October 31st, 1929.*)

The phenomena of the electric response of non-medullated crustacean nerve have been investigated by Levin [1927] and Furusawa [1929]. Meyerhof and Schultz [1929] have studied the oxygen consumption and carbon dioxide production of the non-medullated nerves of several marine invertebrates, among them those of *Maia*. Hill [1929] has measured the heat production of *Maia* nerves during activity. He discusses the probable relationship of his findings to the metabolic processes concerned, and at his suggestion the writer investigated the carbohydrate metabolism of these nerves. A previous series of experiments, performed in 1927 on the ventral abdominal ganglia, is also reported.

The experiments must be regarded as being of a preliminary nature only. The material was obtained, and the first part of the experiments carried out, at Plymouth; circumstances dictated that the actual chemical manipulations should be done at Cambridge. This arrangement necessitated that the technique of the experiments should be planned beforehand; it was naturally based on previous experience with medullated nerve [Holmes and Gerard, 1929], but the results show that the plan adopted suffered from very serious shortcomings. The results can be taken as illustrating only crudely the metabolic events taking place in the non-medullated nerve fibre; even so, the contrast between the results of this work and that of previous experiments with medullated nerve seem sufficiently striking to justify publication in a brief form.

The nerves used were the leg and claw nerves of *Maia*, and the claw nerves of *Cancer*. Portions of leg nerve may readily be obtained, in an undamaged condition, by Furusawa's [1929] technique; such portions weigh, however, about 30 mg., a quantity far too small for lactic acid estimations. Dissection of leg and claw nerves of *Maia* takes some time, and involves (at least in the hands of an unpractised operator) considerable risk of damage to the nerve. The claw nerves of *Cancer* can, on the other hand, be dissected rapidly, and fairly safely, and weigh, usually, between 200 and 300 mg.; they are, however, relatively thick, and it is doubtful whether the isolated nerve receives an adequate oxygen supply, even when kept in the pure gas.

The experimental procedure was as follows. The nerves of the two claws of a crab were dissected out as rapidly as possible, blotted lightly with filter paper, and weighed. One was immersed at once in 98 % alcohol ("initial"), the other was kept either in oxygen or nitrogen at 17–19° for varying periods.

The nerves to be kept in oxygen were arranged on the side of large Thunberg tubes, a little water in the bottom of which served to convert them into moist chambers; after evacuation at the pump, they were filled with oxygen. For the study of the effect of anaerobic conditions, sometimes the nerves were arranged exactly as for the aerobic experiments, except that nitrogen, purified by passage through alkaline hydrosulphite, was used to fill the tube in place of the oxygen. In other experiments, the nerves were immersed in 0·5 cc. of sea water containing urea and $NaHCO_3$, as employed by Meyerhof and Schultz [1929]. This last arrangement gave the most satisfactory results.

Estimations of glycogen, "free carbohydrate" and lactic acid were carried out both on the initial and final samples. As explained in a previous communication [Holmes and Gerard, 1929], glycogen has been found by Eggleton to be soluble in 60 % alcohol; stronger alcohol cannot be relied upon to extract free carbohydrate properly, so that glycogen and free carbohydrate have to be estimated in separate samples, though lactic acid may be estimated in all.

The procedure adopted was similar to that formerly described [Holmes and Gerard, 1929] except that it was possible to apply the Hagedorn and Jensen [1923] technique directly to the glycogen hydrolysates, without using the Bissinger copper-lime precipitation, and, owing to the absence of the medullary sheath, extraction with chloroform was unnecessary. The free carbohydrate fractions were hydrolysed, for 2 hours, with 1·5 % HCl before estimation.

Obviously, it is only possible to obtain a picture of events by these means if the figures for glycogen and free carbohydrate are sufficiently near together to permit of valid average figures being obtained. This is the case with medullated nerve; unfortunately, with non-medullated nerve, the individual variations are so great as to deprive the averages of any significance.

Table I. *Rest in nitrogen.*

All values mg. per 100 g. fresh tissue.

	Initial			Final					
No.	Glycogen	Free carbohydrate	Lactic acid	Glycogen	Free carbohydrate	Lactic acid	Time (hrs)	Temp.	Animal used
14	—	206	103	—	343	263	8·5	18°	*Cancer*
24	—	216	95	—	482	251	8·0	17°	,,
16	—	239	142	—	251	321	19·5	19°	,,
15	1959	—	228	446	—	392	8·0	19°	,,
23	2192	—	127	301	—	315	8·5	17°	,,
10	340	—	—	157	—	—	15·0	17°	,,
17	299	—	80	186	—	279	16·0	19°	,,

The results are set out in Table I. They show, in the first place, that the glycogen and free carbohydrate content of these nerves is enormously greater than that of medullated nerve (medullated nerve; average glycogen content 59 mg. per 100 g., average free sugar content 42 mg. per 100 g.). Meyerhof and Schultz [1929] give the water content of *Maia* nerve as 88 %, so that, in

Exps. 15 and 23, 20 % of the total solids of the nerve must have been glycogen. The glycogen content falls very rapidly under anaerobic conditions; it cannot be stated definitely from the data that it all reappears as free carbohydrate and lactic acid, but considerations, to be mentioned immediately, make it likely that this is the case.

Compared to the carbohydrate reserves, the lactic acid content is very small, though it increases nearly threefold during anaerobiosis.

Table II. *Rest in oxygen.*

All values mg. per 100 g. fresh tissue.

	Initial			Final					
No.	Glycogen	Free carbohydrate	Lactic acid	Glycogen	Free carbohydrate	Lactic acid	Time (hrs)	Temp.	Animal used
5	—	112	120	—	57	163	18	18°	*Cancer*
20	—	182	49	—	130	57	15	18°	,,
22	—	222	120	—	569	110	11	16°	,,
6	542	—	110	371	—	94	15	19°	,,
4	455	—	109	580	—	105	9	16°	*Maia*
18	215	—	73	165	—	176	19	18°	*Cancer*
21	1266	—	215	780	—	105	12	20°	,,

In oxygen (Table II), the fall in glycogen is markedly less than that observed in nitrogen; likewise there is a smaller rise in free sugar. It is questionable whether the relatively thick nerves were properly supplied with oxygen, even when kept in the pure gas, and it may well be that, with thinner nerves, there would have been no fall in the glycogen content. The production of lactic acid is definitely inhibited, but there is nothing to suggest the removal of preformed lactic acid.

Attempts were made to investigate the effects of stimulation; the nerve was dissected free from the lower two segments, and stimulated at intervals of 5–15 minutes with tetanic stimuli of 5 seconds' duration from a Harvard coil, the movements of the claw being taken as an index of effective stimulation. Unfortunately, in no case was stimulation effective for more than 30 minutes, and any change which took place would have been too small to detect in the presence of the large amounts of carbohydrate which have been found to be present in the nerve. This rapid failure of the nerves was at that time observed by the other workers in the laboratory who were using the same material, and was attributed to the extremely hot weather (room temperature 22°). It is apparently a common experience at Plymouth that the tissues of marine animals survive very badly if the temperature of the air and storage tanks rises to any considerable extent. It cannot therefore be claimed that, in the case of the "resting" experiments, the nerves were physiologically active throughout the period of the experiment. On the other hand, many of the earlier experiments, where the nerves were much handled, or were kept for considerable periods in sea water, before use, showed no chemical changes at all. If these conditions are avoided, clearly certain biological processes continue, even though conduction may be impossible.

Table III. *Ventral abdominal ganglia of* Maia.

All values mg. per 100 g. fresh tissue.

	Initial				Final				
No.	Glycogen	Free carbohydrate	Lactic acid*	Total	Glycogen	Free carbohydrate	Lactic acid*	Total	Procedure
1	440	—	30	—	212	—	69	—	20 hrs in H_2 at 17°
2	800	1387	79	2266	245	1362	169	1776	20 hrs in H_2 at 17°
3	1088	1168	37	2293	245	1916	80	2241	20 hrs in H_2 at 18°
4	351	776	37	1164	131	836	99	1066	20 hrs in H_2 at 18°
5	578	806	25	1409	94	1374	102	1570	20 hrs in H_2 at 17°
6	150	1640	61	1851	138	1737	59	1934	"Initial" 2 hrs 20 mins. in hydrogen. "Final" as initial, then 7 hrs in O_2

* Estimated by method of Meyerhof [1920].

In Table III are given the results of a series of experiments, performed in 1927, on the ventral abdominal ganglia of *Maia*. Several crabs (2 to 8, depending on size) were used for each experiment, and the amount of tissue obtained was 2–4 g. Each ganglion was cut in half with sharp scissors, one half helping to make up the "initial" sample, the other the "final" sample. The material was lightly blotted before weighing. The initial samples were placed in alcohol, and worked up immediately; the final samples were placed in large Thunberg tubes, containing 5 cc. of sea water. The tubes were evacuated and filled with hydrogen. They were kept for 20 hours at room temperature and then worked up exactly as were the initial samples. At that time, it was not realised that glycogen was lost during the extraction with 60 % alcohol; the figures for glycogen are, therefore, rather too low, and those for free carbohydrate too high, by a corresponding amount. In spite of this, the figures show clearly that during anaerobiosis glycogen breaks down to give free carbohydrate and lactic acid, and that all the glycogen disappearing can be accounted for, mostly as free carbohydrate and a little as lactic acid.

Table IV. *Values for free carbohydrate before and after hydrolysis.*

All values mg. per 100 g. fresh tissue.

	Initial			Final		
No.	Unhydrolysed	Hydrolysed	Rise %	Unhydrolysed	Hydrolysed	Rise %
2	847	1387	64	1268	1362	7
3	827	1169	41	1626	1916	18
4	519	776	49	845	836	0
5	708	806	14	1347	1374	2
6	—	—	—	1347	1640	22

Table IV shows that there is a further change during anaerobic survival, for hydrolysis of the free carbohydrate fraction gives rise to a much greater increase in reducing value in the case of the initial than in the final samples. The experiments are those already referred to in Table III, where the "hydrolysed" values are given as free carbohydrate.

From the glycogen hydrolysates of *Maia* nerve ganglia, an osazone was prepared, identical in appearance with glucosazone, and melting at 205° after one recrystallisation. The nerve-glycogen hydrolysates, and the free carbohydrate fractions of the ganglia, also yielded typical glucosazone crystals. This appears to afford convincing proof of the identity of the substances estimated.

The point which these experiments brings out most clearly is the very marked contrast in chemical make-up between the medullated nerve tissues of the mammalian central nervous system, and the non-medullated nerves and ganglia of invertebrates.

In view of the large amounts of carbohydrate in non-medullated nerve, the detection of the small metabolic oxidative processes is likely to present great difficulty.

Summary.

1. The peripheral nerves and nerve ganglia of *Maia* and *Cancer* are extremely rich in carbohydrate, which is present as glycogen and as "free carbohydrate." In the case of the ganglia, some, at least, of the carbohydrate is present as di- or poly-saccharide, soluble in 60 % alcohol, and in Schenk's reagent.

2. In nitrogen there is hydrolysis of glycogen, and, in the case of the ganglion, of the soluble di- or poly-saccharide; there is also formation of lactic acid.

3. In oxygen, the formation of lactic acid is inhibited; the breakdown of glycogen is less than occurs in nitrogen.

To my friend, C. F. A. Pantin, my gratitude is due for having originally drawn my attention to the possibilities of working with *Maia* ganglia, and for much help in obtaining material.

I would express my sincere thanks to Dr E. J. Allen, F.R.S., for permitting me to undertake these experiments at Plymouth, and to Prof. A. V. Hill, F.R.S., for helpful advice and criticism.

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CXXXI. THE BACTERIAL DECOMPOSITION OF FORMIC ACID.

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(*Received October 31st, 1929.*)

The bacterial decomposition of formic acid is a subject which, in spite of its apparent simplicity, still presents unsolved problems; it was in the hope of elucidating some of these that the present study was undertaken.

The earliest observation on the subject was made by Hoppe-Seyler [1875]. Later Pakes and Jollyman [1901] studied the anaerobic decomposition of formates by bacteria in a state of active multiplication in meat broth. They collected the mixture of gases formed day by day and analysed it, and, after estimating the carbon dioxide left in the medium, found that the ratio of hydrogen to carbon dioxide was exactly unity, indicating the simple reaction

$$H.COOH = H_2 + CO_2.$$

They did not estimate the amount of formic acid that disappeared, but state that, beginning with 0·2 % to 2·0 % formic acid, roughly 25–30 % was decomposed. From among upwards of thirty species of bacteria used, all those producing gas from glucose also decomposed formic acid anaerobically, and all those producing no gas failed to decompose formic acid. Pakes and Jollyman did not show, however, whether failure to effect this anaerobic decomposition of formic acid involves also an inability to bring about an aerobic breakdown to carbon dioxide and water.

The oxidation of formic acid.

In their studies on the dehydrogenating activity of bacteria Quastel and Whetham [1925] showed that in the presence of methylene blue formates are rapidly dehydrogenated by bacterial suspensions, the rate of reduction of methylene blue by formates under standard conditions being greater than that of any other substance tried with the exception of the sugars. When molecular oxygen replaces methylene blue as hydrogen acceptor the relatively high rate of oxidation of formate, as compared with that of other substances, is no longer shown, the velocity of its oxidation, in fact, not attaining that of lactate. Measurements of oxygen uptake show that in the case of *B. coli communis* the oxidation is complete, that is, proceeds according to the equation [Cook and Stephenson, 1928]

$$H.COOH + O = H_2O + CO_2.$$

Arising out of these observations, it is of interest to know whether the

anaerobic mechanism studied by Pakes and Jollyman, whereby certain bacterial species when growing on broth decompose formic acid according to the equation

$$H.COOH = H_2 + CO_2,$$

is identical with the aerobic mechanism studied by Cook and Stephenson, by which suspensions of dead bacteria in phosphate buffer decompose formates according to the equation

$$H.COOH + O = H_2O + CO_2.$$

The preparation of formic dehydrogenase.

The first step in the attack on this problem was an attempt to make a cell-free preparation of formic dehydrogenase in a manner analogous to that used in the preparation of lactic dehydrogenase from *B. coli* [Stephenson, 1928] and from yeast [Bernheim, 1928], the citric dehydrogenase from liver [Bernheim, 1928] and the succinic dehydrogenase from muscle [Ohlsson, 1921]. Autolysis of the cells in a buffer solution proving unsuitable for the purpose, the more drastic method of digesting them with trypsin was resorted to. A suspension of *B. coli* was prepared by growing the organism on plates about 6 in. in diameter containing tryptic broth agar to which 0·5 % sodium lactate had been added; the organisms were grown for 24 hours, washed off the plates with dilute phosphate buffer at p_H 6·0, centrifuged, and washed three times with Ringer's solution. The growth from 24 plates was suspended in about 100 cc. of Ringer's solution and aerated for half an hour to remove traces of oxidisable materials. The digestion was carried out by adding 5 cc. of Benger's *liquor pancreaticus* to 100 cc. of suspension, buffering the mixture at p_H 7·6, and incubating at 37°; it was found that sodium fluoride had no effect on the action of the digested preparation on formic acid, and 1 % sodium fluoride was therefore added to prevent growth of contaminating organisms.

At intervals during the incubation samples were withdrawn and tested to ascertain the reduction time of methylene blue by the three hydrogen donators formate, succinate and lactate. It was found that the activity of these three dehydrogenases was greatly increased by digestion with trypsin; the succinic and lactic dehydrogenases, however, soon disappeared (being very susceptible to the action of fluoride) but the formic dehydrogenase was stable and attained five times its original activity (Fig. 1). When this very active digested suspension was centrifuged and filtered through a glass filter (G 4), about 50–60 % of its activity was found in the filtrate; for instance, in one experiment the following reduction times were obtained:

	Reduction time mins.	secs.
Original suspension	25	20
Suspension digested for 4 weeks ...	5	40
Supernatant fluid after centrifuging	7	45
Residue	25	0
Filtrate through glass filter ...	10	0

The filtrate, examined under the microscope, was entirely free from whole cells, but contained large quantities of small particles of cell débris from which

the enzyme could not be separated; by repeated centrifuging at high speed a supernatant fluid was obtained which was almost inactive, while the small quantity of residue, made up to the same volume, possessed almost all the activity of the original filtrate.

	Reduction time mins.	secs.
Filtrate	3	10
Final supernatant fluid	36	0
Residue	3	20

Filtration through a Seitz filter and through a layer of kieselguhr gave similar results. The formic dehydrogenase was not therefore obtained in the same soluble state as Stephenson's lactic dehydrogenase, but a very active preparation free from whole cells was made.

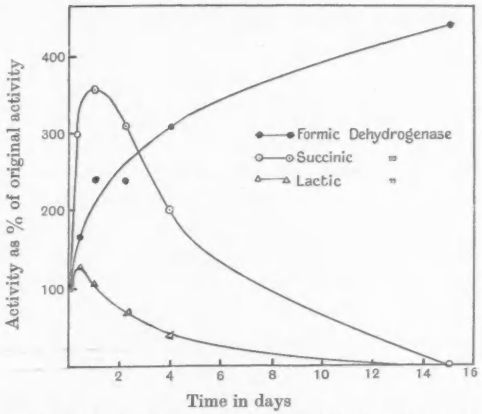

Fig. 1. Effect of tryptic digestion on the activity of the dehydrogenases of *B. coli*. (Reciprocals of reduction times.)

The *liquor pancreaticus* used in the digestion, besides containing trypsin, was rich in amylase, and it was thought advisable to determine which of these two enzymes was producing the effects described. By taking the *liquor pancreaticus* to p_H 3·0 by cautious addition of hydrochloric acid, and restoring it to neutrality after 10 minutes at room temperature, it was possible to destroy more than 99·5 % of the amylase, while leaving more than 70 % of the trypsin. Two digestions, carried out simultaneously with treated and untreated *liquor pancreaticus*, gave practically identical results with regard to the increase in activity of the formic dehydrogenase, so the result may be attributed entirely to the action of the trypsin.

It is clear from these experiments that the effect of tryptic digestion on a suspension of *B. coli* is to increase the activity of the formic dehydrogenase (as tested by the methylene blue technique), while eliminating the other dehydrogenating mechanisms of the cell. The next point was to ascertain whether the formic dehydrogenase preparation thus obtained was capable of

oxidising formic acid by means of molecular oxygen. This was done by measuring the oxygen uptake of the enzyme with formic acid in a Barcroft apparatus, the two cups containing:

Left-hand cup	Right-hand cup
1 cc. enzyme preparation	1 cc. enzyme preparation
1 cc. phosphate buffer, p_H 6·0	1 cc. phosphate buffer, p_H 6·0
1 cc. water	1 cc. formic acid (M/40 or M/100)

The results obtained were as follows (mm.^{3} O_2):

M/100 formic acid	M/40 formic acid
22	20
18	16
23	16
18	21
Theoretical = 112	Theoretical = 280

When a fresh suspension of *B. coli* was used, the oxygen uptake corresponded to complete oxidation, *e.g.*

with M/40 formic acid	291 and 294 mm.^{3} O_2	(theoretical = 280);
with M/20 formic acid	523	(theoretical = 560).

It has previously been shown by Stephenson that lactic acid is not oxidised aerobically by lactic dehydrogenase alone, but in the presence of an autoxidisable dye such as methylene blue is oxidised completely to pyruvic acid. Since the formic dehydrogenase preparation was so active in reducing methylene blue, it seemed probable that addition of the dye to the Barcroft cup would enable the system to use molecular oxygen to oxidise the formic acid, but in this case the oxygen uptake remained unaltered.

M/40 formic acid + 0·5 cc. 1/5000 methylene blue	32 and 19 mm.^{3} O_2.

It now remained to account for the small constant uptake, corresponding to oxidation of 0·0006 M formic acid. The amount, as is shown by the figures given above, was independent of the concentration of formic acid; it therefore appeared possible that on oxidation a substance was formed which, when its concentration reached a certain value, inhibited further enzyme action. Products of the reaction which might cause this inhibition were then sought for; oxalic acid, formaldehyde and peroxide were tried. The first two were found to have no effect, either on the activity of the formic dehydrogenase preparation (tested by the methylene blue technique) or on the oxidation of formic acid by a fresh suspension of *B. coli*, even when used in a concentration greater than could possibly be present in these experiments, viz. oxalic acid N/100, formaldehyde M/10. Peroxide could not be detected in the contents of the Barcroft cups by the titanium and guaiacum tests, whilst hydrogen peroxide in a concentration of 0·0003 %, which is easily detectable by these tests, caused no inhibition of the dehydrogenase. If these have no effect on the dehydrogenase it may be assumed that they do not affect the oxygen uptake in the presence of methylene blue. Bernheim [1928] attributed the inability of the soluble citric dehydrogenase to take up oxygen, even in the presence of methylene blue, to destruction of the enzyme by mere shaking in

air. This explanation cannot be applied in the case of the formic dehydrogenase, for samples which have been shaken alone in a Barcroft cup for 2 hours show only a small decrease in activity, while samples shaken in the presence of formic acid are completely inactivated, *e.g.*

		Reduction time mins.	secs.
Original preparation		2	50
After 2 hours' shaking without formic acid	(1)	6	25
	(2)	6	0
After 2 hours' shaking with formic acid ...	(1)	>60	0
	(2)	>60	0

The only method by which the formic dehydrogenase could be shown to oxidise completely a known quantity of formic acid was by causing it to reduce an equimolecular amount of methylene blue, and measuring the oxygen required for re-oxidation of the leuco-methylene blue. Barcroft apparatus were set up containing:

Left-hand cup	Right-hand cup
1 cc. enzyme preparation	1 cc. enzyme preparation
0·5 cc. buffer, p_H 6·0	0·5 cc. buffer, p_H 6·0
1 cc. $M/20$ methylene blue	1 cc. $M/20$ methylene blue
0·5 cc. water	0·5 cc. $M/20$ formic acid

To determine the time required for complete oxidation of the formic acid, a Thunberg tube was prepared with 1 cc. enzyme preparation, 0·5 cc. buffer, 0·5 cc. $M/20$ methylene blue, 0·5 cc. $M/20$ formic acid and 0·5 cc. water; the time taken for reduction of the methylene blue in this tube gave an approximation to the time required in the Barcroft experiment. The Barcroft apparatus was evacuated and placed in the bath at 40° for a rather longer time than that found by the above method, and then air saturated with water vapour at 40° was admitted to both cups. The oxygen uptake was then measured in the usual way, and was found to be of the right order for complete oxidation (Fig. 2, p. 1192):

0·5 cc. $M/20$ formic acid

	264 mm.^{3} O_2
	366
	284
Mean	305

Theoretical = 280

It thus appears that treatment of the cells with trypsin has increased the dehydrogenase activity, but decreased the capacity of the cell to oxidise formic acid by molecular oxygen.

The action of the formic dehydrogenase preparation in liberating molecular hydrogen from formates was next investigated. Experiments were made in test-tubes containing Durham tubes, formic acid (0·01–0·1 M), phosphate buffer (p_H 5·0–9·0), and the enzyme, these were incubated anaerobically. In no case was any detectable hydrogen liberated. It thus seems that tryptic digestion of *B. coli* suspensions, though increasing the activity of the dehydrogenase towards methylene blue, has resulted in the elimination of the mechanism liberating molecular hydrogen anaerobically.

Additional evidence as to the identity or non-identity of the aerobic and anaerobic mechanisms for the decomposition of formic acid was then sought for by the study of an organism in which the latter mechanism is known to be absent. For this purpose *B. typhosus* was selected.

Presence of formic dehydrogenase in B. typhosus.

A suspension of *B. typhosus* was prepared in the same way as in the case of *B. coli*. The reduction times with methylene blue were as follows:

	Reduction time (mins.)
Blank (no hydrogen donator)	100
Formic acid *M*/20	$5\frac{3}{4}$
Succinic acid *M*/20	17
Lactic acid *M*/20	$3\frac{1}{2}$

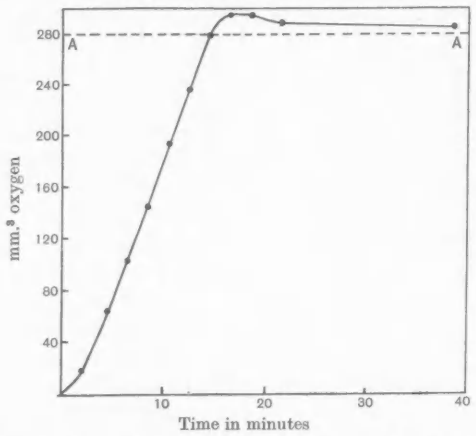

Fig. 2. Oxygen uptake of leuco-methylene blue produced by oxidation of formic acid by the dehydrogenase preparation. *A* represents the theoretical amount.

Hence *B. typhosus* shows a marked power of activating formic acid as a hydrogen donator to methylene blue. This organism was also sown into test-tubes containing Durham tubes with tryptic broth and 0·5 % formic acid; no gas was produced after 3 days' incubation, while similar tubes sown with *B. coli* produced much hydrogen in 24 hours. This result is in accordance with Pakes and Jollyman's findings for non-gas-producing organisms. Thus it appears that in the case of *B. typhosus* we have an example of an intact organism possessing the mechanism for dehydrogenating formates but lacking the mechanism for the anaerobic decomposition of formic acid into carbon dioxide and molecular hydrogen. In this respect the intact cell of *B. typhosus* resembles the digested cell of *B. coli*.

THE COURSE OF THE ANAEROBIC DECOMPOSITION OF FORMIC ACID.

Preliminary experiments showed that without strong buffering the reaction was stopped by the increasing alkalinity; phosphate in greater concentration

than $M/10$ also inhibited the reaction. Finally $M/10$ phosphate buffer at p_H 6·0 and $M/10$ formic acid were used, the p_H after complete decomposition of the formic acid being about 9.

The formic acid, buffer solution and suspension of washed and aerated cells of *B. coli* were put into a 250 cc. flask which was placed in a water-bath at 37° and connected to a wash-bottle to prevent leakage of oxygen into the flask. Oxygen was supplied through a T-piece placed between this wash-bottle and two drying tubes containing calcium chloride, which were followed by a tube containing a platinum spiral for the combustion of hydrogen, weighed calcium chloride tubes for the estimation of the water formed and baryta tubes for the estimation of carbon dioxide.

The flask was first washed out with a rapid current of oxygen-free nitrogen. Samples for the estimation of formic acid were withdrawn every 24 hours through a capillary tube, bent through 180° at the top and carrying a tap; these estimations were carried out by a method based on that described by Dakin [1913]. The sample (containing 5–10 mg. of formic acid) was diluted with 30 cc. of 50 % alcohol, acidified with phosphoric acid and distilled *in vacuo* into excess of ice-cooled sodium hydroxide; the residue was redistilled with a further 20 cc. of 50 % alcohol. The combined distillates were evaporated to about 10 cc., placed in a pressure bottle, slightly acidified with acetic acid, and heated with a twenty-fold excess of saturated mercuric chloride solution for 7 to 8 hours in a boiling water-bath, the precipitated calomel being collected and weighed. Each estimation was carried out in duplicate, these agreeing to within 1–2 %. Known quantities of formic acid gave yields of only 98–99 % of the theoretical amount of calomel.

The amounts of hydrogen and carbon dioxide formed during each period of 24 hours were estimated by blowing 2 litres of oxygen-free nitrogen through the apparatus in the course of about 2 hours. With an apparatus of the size used, this would mean that, assuming complete mixing of the gases, 99·98 % of the gas in the flask had been washed over. The hydrogen was measured by adding an excess of oxygen, drying the mixture, passing it over the heated platinum spiral and weighing the water collected in the calcium chloride tubes. The carbon dioxide was estimated in the residual gas by passing it through the series of bottles containing standard baryta and titrating the remaining baryta. The apparatus was tested by passing moist air through the series of tubes; no increase in the weight of the calcium chloride tubes was found. Two litres of air containing 1·84 % of hydrogen (previously estimated on the Haldane apparatus) gave 0·0310 g. of water, corresponding to a yield of 104 %.

Results.

After a preliminary period of about 24 hours, during which very little decomposition took place, the reaction proceeded at a uniform rate for several days, and then decreased in velocity (Fig. 3). A possible interpretation of the shape of the curve will be discussed later; these results serve to show, however,

that at every stage the quantities of hydrogen and carbon dioxide formed correspond to the amount of formic acid decomposed, according to the equation

$$H.COOH = H_2 + CO_2.$$

The production of hydrogen in aerobic conditions.

In Cook and Stephenson's work on the oxidation of formic acid by *B. coli* the amount of oxygen taken up in a Barcroft apparatus was exactly that required by the equation

$$H.COOH + O = H_2O + CO_2;$$

if any hydrogen had been produced their results would have been too low. In order to find out whether in aerobic conditions any hydrogen can be formed Barcroft apparatus were set up containing:

Left-hand cup	Right-hand cup
1 cc. suspension of *B. coli*	1 cc. suspension of *B. coli*
0·5 cc. buffer, p_H 6·0	0·5 cc. buffer, p_H 6·0
1 cc. water	1 cc. 0·4 *M* formic acid
0·5 cc. tryptic broth	0·5 cc. tryptic broth

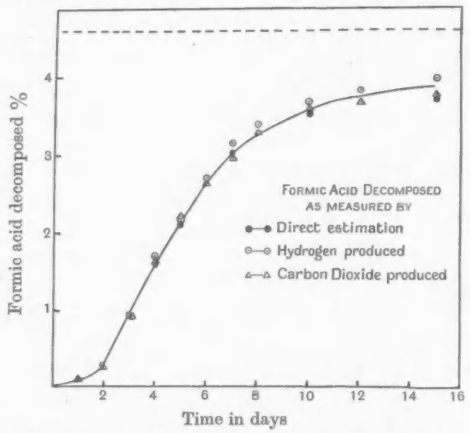

Fig. 3. The course of the anaerobic decomposition of formic acid.

The tryptic broth was added because the production of hydrogen is always more rapid in the presence of broth. After the apparent oxygen uptake had reached 2750 mm.^{3}, corresponding to oxidation of about 60 % of the formic acid, the rate of oxidation slowed down, and at this point 10 cc. of the gas in the right-hand cup were transferred to a Haldane apparatus. Analysis of the gas showed that it contained no detectable amount of hydrogen (*i.e.* less than 0·02 %), demonstrating that less than 0·15 % of the total formic acid (taking the volume of the Barcroft cup as roughly 40 cc.) had been decomposed according to the equation

$$H.COOH = H_2 + CO_2.$$

A state equivalent to the aerobic condition can be produced by adding

various hydrogen acceptors other than oxygen to the medium, *e.g.* nitrate and fumarate. Experiments with these substances were carried out in test-tubes with Durham tubes; each tube contained 0·1 *M* formic acid, 0·1 *M* phosphate buffer at p_H 6·0, tryptic broth and 0·1 *M* potassium nitrate or sodium fumarate, and all were sown from a fresh culture of *B. coli*. In the absence of a hydrogen acceptor growth was good and large amounts of gas were produced; in the other tubes the growth was equally good, but in the presence of nitrate no gas was produced at all, and in the presence of fumarate a very small amount of gas was usually observed.

It may therefore be concluded that in thoroughly aerobic conditions the decomposition of formic acid into hydrogen and carbon dioxide is entirely suppressed, its place being taken by the oxidising reaction.

Discussion.

The obvious explanation of the mechanism of the decomposition of formic acid is that the primary reaction is the splitting of the molecule into hydrogen and carbon dioxide; the hydrogen is then, in the absence of a hydrogen acceptor, liberated in the molecular state, or, in the presence of a hydrogen acceptor such as oxygen, nitrate or methylene blue, oxidised to water or leuco-methylene blue. It is found, however, that *B. coli* cells, after digestion with trypsin, although still capable of actively dehydrogenating formic acid, can no longer decompose it into hydrogen and carbon dioxide; similarly *B. typhosus* can dehydrogenate formic acid, but cannot liberate hydrogen anaerobically. This leads one to suppose that in *B. coli* there are two distinct mechanisms for the decomposition of formic acid, one anaerobic and the other aerobic, of which only the latter is present in *B. typhosus*. If this were so, one would expect to find that, even in thoroughly aerobic conditions, a part of the formic acid (provided that its concentration is well above that required for saturation of the dehydrogenase) will be decomposed into hydrogen and carbon dioxide. Experiment shows that, as far as can be detected by the method employed, thorough aeration completely suppresses the formation of hydrogen, showing that there is after all some connection between the two processes. One finally reaches the conclusion that the process common to both reactions is the activation of the hydrogen atoms of the formic acid by the dehydrogenase, and that for the union of the two atoms to form molecular hydrogen a further enzyme mechanism is necessary, which is found only in certain organisms, and which does not operate in the presence of hydrogen acceptors.

The relation of dehydrogenation and of hydrogen production to the viability of the organisms.

It has been shown previously [Cook and Stephenson, 1928] that the bacterial oxidation of lactate and formate is independent of the number of living cells present. Since an experiment on the production of hydrogen lasts at least a week, so that the surviving cells have ample opportunity of growing

on the products of autolysis of the dead ones, it was advisable to ascertain whether the production of hydrogen was dependent on the viability by following the change in the number of viable cells during the course of an experiment. The counting was carried out by the method described by Wilson [1922], and the formation of hydrogen was followed in Durham tubes containing buffer at p_H 6·0, 0·5 % formate and the suspension of *B. coli*. This method could be made roughly quantitative by simply measuring the length of tube occupied by the gas, and by taking the mean of six similar tubes curves were obtained of the same shape as those given by the more accurate method already described. The gas in the Durham tubes was in a number of cases analysed in the Haldane apparatus and was found to be of very nearly constant composition, viz. 20 % carbon dioxide and 80 % hydrogen.

The results showed that when a suspension of *B. coli* is incubated with formate and buffer, the viable count decreases rapidly during the first 24–48 hours, while no gas is given off, and during the subsequent evolution of hydrogen remains practically constant, either slightly decreasing (Fig. 4) or slightly

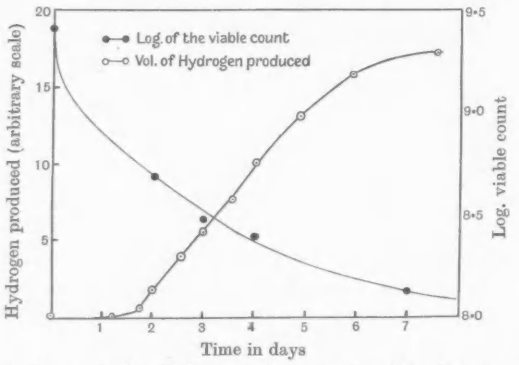

Fig. 4. Changes in the viable count of a suspension of *B. coli* during the decomposition of formic acid into hydrogen and carbon dioxide.

increasing (Fig. 5). If the suspension is first treated with ultra-violet light till the viable count is reduced to about one-millionth of its original value, then on incubation with formate the cells multiply steadily until, after about 4 days, the count is roughly equal to that attained at the end of the experiments with untreated cells; at this point the gas production begins and the count remains almost stationary (Fig. 6). These results suggest that the decomposition of formic acid into hydrogen and carbon dioxide depends not merely on the number of viable cells, but on the rate of their multiplication, for in each case the hydrogen is given off when the rate of multiplication is at its maximum, that is, when the death-rate is balanced by the division of the survivors.

Two other pieces of evidence seem to support this view; the first is that in the presence of tryptic broth, where the cells can multiply very rapidly, the

decomposition of formic acid is very much more rapid than in the absence of broth. The second is that when various concentrations of certain growth-inhibiting substances were added to the solution, a smaller concentration was required to prevent hydrogen production by a suspension of *B. coli* than was required to prevent growth of the organism in broth, *e.g.*

	%	Growth	Hydrogen
$FeCl_3$	0·032	+ +	+
	0·064	+	+
	0·128	−	−
$ZnSO_47H_2O$	0·12	+ +	+
	0·18	+ +	+
	0·27	−	−
$NiSO_47H_2O$	0·045	+ +	+
	0·068	+ +	−
	0·101	−	−
NaF	0·1	+ +	+
	0·24	+ +	−
	0·53	+	−

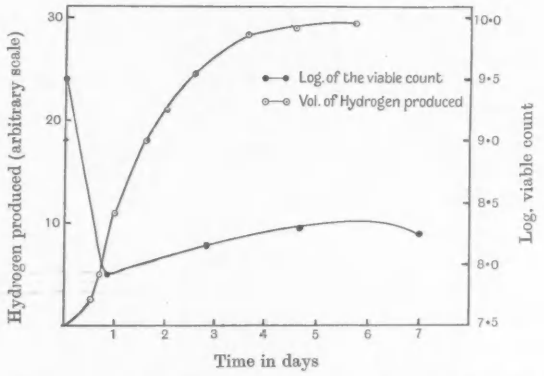

Fig. 5. Changes in the viable count of a suspension of *B. coli* during the decomposition of formic acid into hydrogen and carbon dioxide.

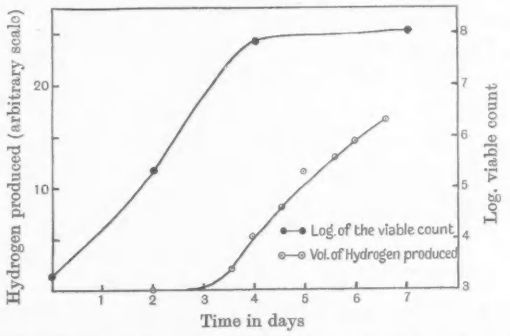

Fig. 6. Changes in the viable count of a suspension of *B. coli* that had been treated with ultra-violet light, during the decomposition of formic acid into hydrogen and carbon dioxide.

All that can be said definitely on this subject at present is that no hydrogen has been obtained in conditions in which growth of the organism was prevented, and that whenever hydrogen was produced multiplication of the organisms could be demonstrated.

Summary.

1. Tryptic digestion of a suspension of *B. coli* cells causes a transitory increase in the activity of the succinic and lactic dehydrogenases, and a permanent one in that of the formic dehydrogenase to five times its original value. From the product of the digestion a preparation of formic dehydrogenase can be made, which is free from intact cells but contains cell débris.

2. This preparation will not oxidise formic acid by means of molecular oxygen, nor will it decompose it into hydrogen and carbon dioxide anaerobically.

3. In thoroughly aerobic conditions *B. coli* oxidises formic acid completely, giving no free hydrogen.

4. *B. typhosus* reduces methylene blue in the presence of formate, but cannot liberate molecular hydrogen from formate.

5. The bearing of these facts on the relation between the two modes of decomposition of formic acid is discussed.

I wish to thank Sir F. G. Hopkins for his encouragement and Miss M. Stephenson for constant help and advice while this work was being done.

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CXXXII. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. VIII.

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Report to the Medical Research Council.

(*Received October 31st, 1929.*)

Recent contributions to this investigation [Zilva, 1927, 1928] dealt with observations bearing on the stability of the antiscorbutic factor in lemon juice. Two phenomena were recorded in this connection; (*a*) the presence of a reducing principle, the destruction of which preceded the inactivation of the vitamin, and (*b*) the modification in the stability of the antiscorbutic activity of decitrated lemon juice when stored in neutral medium after having been autoclaved under strictly anaerobic conditions for 1 hour. This latter change was provisionally attributed to the destruction of a "thermolabile factor" in the process of autoclaving, as a suitable working hypothesis. A number of experiments have been carried out in this direction and as would be expected in the case of such a complex subject, the significance of many of the results cannot be fully interpreted in the light of our present knowledge. Some results, however, have yielded information which is considered of sufficient interest to be recorded, especially as they point to the possibility that the effects of the autoclaving may not be due to the destruction of a stabilising factor but to the formation of a substance or substances which conduce to the acceleration of the destruction of the antiscorbutic factor.

The effect of preliminary heating at temperatures lower than 100° on the stability of the antiscorbutic factor in decitrated lemon juice.

On the assumption that the modification in the autoclaved decitrated lemon juice was due to the destruction of a "thermolabile factor" it was of prime interest to ascertain the effect of heating at lower temperatures in order to establish whether a thermolabile enzyme was involved. With this end in view, the activity of stored solutions of ordinary decitrated lemon juice, previously heated at 55–58° and 80–85° respectively, for 1 hour under anaerobic conditions, was studied.

The actual procedure was as follows. 50 cc. of ordinary decitrated lemon juice adjusted to p_H 7 were placed in an ampoule of about 170 cc. capacity. The vessel was then exhausted under a vacuum pump (1 mm.), until the dissolved gases were no longer evolved. This was followed by the admission of pure

nitrogen and the evacuation and filling with the nitrogen were carried out three times, leaving the ampoule finally filled with nitrogen. After the last admission of nitrogen, a small glass **S**-tube containing a mercury seal was attached to the ampoule which was then immersed in the water-bath adjusted to the required temperature and kept constant by means of a thermostat. Five or ten minutes were allowed for the adjustment of the temperature before the actual commencement of the heating was timed. The nitrogen used was prepared by the action of ammonia on copper turnings and passed through a train of bottles containing sulphuric acid, sodium hydrosulphite and sodium hydroxide. There was little change in p_H after the heating. The solutions, which were prepared daily, were adjusted if necessary to p_H 7 and kept at this reaction during storage in air for 7 days in the cold room. The biological tests were carried out as described by the author elsewhere.

The first set of experiments made it plain that this preliminary anaerobic heating at 55° or 85° did not bring about the same modification in the stability of the antiscorbutic activity as autoclaving had done. It was, however, observed that active decitrated lemon juice heated at these comparatively low temperatures showed a greater vitamin loss before storing than was previously observed in anaerobically autoclaved juice. This unexpected observation made it advisable to repeat the experiment. Consequently five sets of experiments in which the active solution was heated at temperatures of 55–58° and four sets in which temperatures of 80–85° were employed were carried out at different times of the year between June, 1927, and January, 1929, thus affording the opportunity of using lemons of different origin and at different stages of senescence. The results obtained on the whole confirmed those observed in the preliminary experiments. Only in one set in which the juice was heated at 55° was there a suggestion of a higher deterioration of the activity on storing. As 140 guinea-pigs were used in this group of experiments it is not proposed to give the details of all the tests. Fig. 1, however, depicts a representative experiment. It is representative in so far that it shows how the same juice after different treatments behaved on storage. It is seen that the stored autoclaved batches, as has been observed on numerous occasions before, showed hardly any antiscorbutic activity at all in the doses tested, whilst the batches that had been heated at the lower temperature did not deteriorate proportionately more than the unheated juice on storage. It must, however, be pointed out that the higher destruction in the antiscorbutic activity of the solutions heated at 55–58° and 80–85° was more marked in the other sets of experiments and that it has not been traced so far to any fault in the technique.

All the heated and control batches were titrated daily with phenolindophenol before and after storage in order to ascertain the reducibility of the solutions. The destruction of the "reducing principle" on storage in the solutions which were heated at 58° or 85° was of the same order as that of the unheated solutions, whilst in the autoclaved juices the destruction was almost complete after 7 days' storage.

Fig. 1.

D, decitrated lemon juice; A, autoclaved lemon juice; +died; C, chloroformed; S, scurvy, SS, slight scurvy; VSS, very slight scurvy; i, intercurrent disease; N, normal.

These experiments show that, whatever the modification in the stability of the antiscorbutic activity brought about by autoclaving may be due to, the destruction of a thermolabile enzyme or of a thermolabile link in an enzyme system is not the cause of it.

The effect of the addition of decitrated juice inactivated by aeration on the stability of autoclaved decitrated juice.

It was next of interest to ascertain whether the addition of decitrated lemon juice previously inactivated by aeration at ordinary temperature [Zilva, 1922] could replace the hypothetical "thermolabile factor." To a decitrated lemon juice autoclaved anaerobically, an equal volume of the same juice previously aerated at room temperature for 7–8 hours, which was found by a parallel control test to be antiscorbutically inactive, was added. On testing the equivalents of the usual doses (three animals on a dose), it was found that this addition in no way influenced the deterioration of the antiscorbutic activity of the autoclaved solution.

Attempts to fractionate the supposed "thermolabile factor."

Two fractions were employed. One was the antiscorbutically inactive precipitate obtained by the treatment of ordinary decitrated lemon juice with alcohol and the other was the neutral lead acetate fraction from the same source. In the first case decitrated lemon juice was concentrated to about one-tenth of its original volume. On addition to this concentrated solution of a large excess of alcohol, a precipitate which was soluble in water was formed. After filtration on a Büchner funnel, the filter paper and precipitate were introduced into anaerobically autoclaved decitrated lemon juice and the precipitate dissolved. A precipitate equivalent to two volumes of the decitrated juice was introduced into each volume of the autoclaved solution. These solutions were prepared daily and stored as before for 7 days in the cold room at p_H 7 before testing. Besides this preparation, the redissolved (in water) precipitate and the autoclaved decitrated lemon juice not containing the fraction were tested as controls. The precipitate was, as usual, found to be inactive and the addition of it to the autoclaved solution did not in any way compensate for the modification in the stability of the antiscorbutic activity produced by autoclaving.

The investigation of the compensating property of the neutral lead acetate fraction was complicated by the fact that in this instance the fraction retained some antiscorbutic activity. It has been pointed out already [Zilva, 1928] that the fraction precipitated from decitrated lemon juice by lead acetate between p_H 5 and 7 does not always contain most of the antiscorbutic activity, as found by the author in his earlier experiments. In fact, during the last two years, this fractionation has been carried out many times and a disturbing number of fractions of low activity has been obtained. It is evident that some still unknown factor, possibly a co-adsorbent, regulates this fractional pre-

cipitation of the antiscorbutic factor by lead acetate. Although the cause has not yet been satisfactorily ascertained, one observation has been made which has a bearing on it, namely, that sometimes—not often in the author's experience—considerable activity is found in the neutral lead acetate fraction, *i.e.* the fraction of p_H below 5·4–5·2. In this experiment the neutral lead fraction which was tested as a control showed some antiscorbutic activity and consequently the autoclaved decitrated juice, to which this fraction was added, was found to be rather more active after 7 days' storage than a similar solution to which it was not added. The results of this imperfect experiment did not, however, suggest that the addition of the fraction compensated for the modification in stability produced by the autoclaving. In view of subsequent developments of the problem, it was not considered expedient to repeat this experiment.

Changes produced in decitrated lemon juice by autoclaving.

While studying the effect of various temperatures on the "thermolabile factor," attention was naturally focussed on the enzyme activity of the heated solutions. It was found that decitrated lemon juice possessed the property of oxidising *p*-phenylenediamine after a certain lag when a suitable quantity of hydrogen peroxide was added. This activity was retained, somewhat weakened, after autoclaving. Other sources of the antiscorbutic factor, such as swede juice, cabbage juice, or tomato juice, behaved similarly in this respect. In lemon juice it was found that the peroxidase activity persisted after the vitamin in it was partially or totally destroyed, thus aerated, stored autoclaved solutions and solutions which were inactivated by being made alkaline retained this enzymic activity. The enzyme was also precipitated by lead acetate partly at p_H 5 and partly at p_H 7.

Another observation made in this connection was that whilst the property of reducing phenolindophenol by active solutions was somewhat reduced by autoclaving under anaerobic conditions, the capacity for decolorising iodine was greatly increased. This increase in the capacity for reducing iodine does not take place when the solution is heated anaerobically at 58° or 85°. As an example illustrating this, the following may be quoted. A sample of decitrated lemon juice 5 cc. of which decolorised 20 cc. of 0·02 % phenolindophenol and 26·5 cc. $N/1000$ iodine quickly and approximately a further 20 cc. of the iodine slowly, after being autoclaved anaerobically, decolorised 15 cc. of phenolindophenol, and 30 cc. of the iodine quickly, after which the rate of decoloration of the iodine became definitely slower and a further 60 cc. of iodine (approximately, end-point not sharp) had to be added before the decoloration ceased.

These observations taken in conjunction with the failure so far to demonstrate the presence of a thermolabile stabilising factor in antiscorbutic solutions directed the writer's view to a possible alternative explanation of the modification in the stability of the antiscorbutic activity produced by autoclaving,

namely, the formation of a substance or substances capable of accelerating the mechanism of inactivation of the vitamin in air.

Experimental evidence so far obtained supports this view. Thus, if equal volumes of autoclaved and unheated decitrated lemon juice are mixed and stored at p_H 7 for 7 days, the capacity for reducing phenolindophenol disappears almost to the same extent as it does in autoclaved juice. This disappearance, as pointed out in a previous communication, is much slower in unheated juice. Fig. 2 gives a graphic representation of this phenomenon. The disappearance of the "reducing principle," which is assumed to have a protective action on the vitamin, has so far been found without exception by the writer to be an indication of the inactivation of the vitamin. A preliminary biological test shows that an acceleration in the destruction of the antiscorbutic principle is indeed brought about by the addition of autoclaved to unheated decitrated lemon juice to about the same extent as by autoclaving.

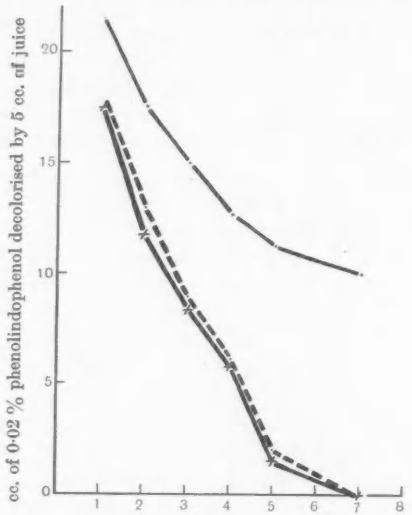

Fig. 2.

——— Unheated juice. – – – Autoclaved juice.
—— × —— Autoclaved + unheated juice.

Another experiment also lent support to the above view. Since the formation of a substance which decolorises iodine suggests the likelihood of its being phenolic in character, the influence of the addition of quinol to unheated decitrated juice was studied. In this case, too, there was a quicker disappearance of the "reducing principle". For instance, 5 cc. of a decitrated lemon juice decolorised 23 cc. of 0·02 % phenolindophenol solution. On storage, this titre fell to 14·0 cc., whilst the stored autoclaved juice of the same batch and the unheated juice to which quinol was added (0·12 %) fell to 3 cc. and 2 cc.

respectively. Preliminary experiments show that also in this case the addition decreases the stability of the antiscorbutic activity of the juice to the same extent as autoclaving.

Whether there be a connection or not between the thermostable peroxidase and the increased power of iodine reduction produced by autoclaving, the possibility of such a connection obviously cannot be dismissed until it is proved to be otherwise. The above experimental evidence, in any case, strengthens the view that the modification in the antiscorbutic stability of decitrated lemon juice by autoclaving is produced rather by the formation of a substance or substances conducing to the destruction of the vitamin than by the destruction of a "thermolabile stabilising factor," as originally assumed. This view is being borne in mind by the writer in his general study of the antiscorbutic factor.

SUMMARY.

1. There is no modification in the stability of the antiscorbutic activity of decitrated lemon juice previously heated for 1 hour under anaerobic conditions at 55–58° or at 80–85° as there is when the juice is autoclaved.

2. The addition of decitrated lemon juice, in which the vitamin has been inactivated by aeration at ordinary temperature to autoclaved juice, does not compensate for the modification in stability, nor does the addition of the fraction obtained from decitrated lemon juice by precipitation with alcohol or with neutral lead acetate.

3. Decitrated lemon juice, cabbage juice, tomato juice and swede juice contain a thermolabile peroxidase.

4. Autoclaving decitrated lemon juice under anaerobic conditions diminishes its capacity for reducing phenolindophenol to some extent, but increases its capacity for decolorising iodine. This increased capacity for decolorising iodine is not observed after the decitrated lemon juice has been heated anaerobically at 58° or 85°.

5. The addition of autoclaved decitrated juice to unheated juice accelerates the destruction of the "reducing principle" and of the antiscorbutic activity in it on storage at p_H 7. The addition of quinol has a similar effect.

6. It is suggested that the modification in antiscorbutic stability of decitrated lemon juice produced by autoclaving may possibly be due to the formation of a substance or substances conducing to the deterioration of the vitamin rather than to the inactivation of a thermolabile stabilising factor.

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CXXXIII. THE DETERMINATION OF THE TOTAL CALCIUM CONTENT OF BLOOD-SERUM.

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(*Received August 21st, 1929.*)

The values given for the normal calcium content of blood-serum vary a good deal. It is therefore advisable, when giving such figures, to mention the method used in their determination.

Various investigators have compared their results with determinations made in the ash of serum.

The idea of the following investigation was as follows.

1. To make determinations according to the method of Trevan and Bainbridge [1926], both in serum and ash, and at the same time in a known standard solution which corresponds as closely as possible with a solution of serum-ash.

2. To ascertain whether the magnesium present in the serum appreciably affects the calcium values.

3. To ascertain in what standard solution comparative tests can be made.

4. To compare the figures found by the Trevan-Bainbridge method with those of some of the permanganate methods.

When ammonium oxalate is added to serum, a precipitate of calcium oxalate is formed, and many methods of determination are based on this reaction.

The objection to precipitating calcium as oxalate is the difficulty of separating it from magnesium. The separation is based on the difference in solubility of the two oxalates. But from solutions in which the limit of solubility of the magnesium oxalate present is far from being reached, magnesium is still occluded by the calcium oxalate that is precipitated [Lemarchand, 1925]. When, therefore, a large amount of magnesium is present, the calcium oxalate may be precipitated twice; a more favourable proportion of the two metals is obtained the second time.

Under certain circumstances, however, even with only one precipitation, a separation to a certain degree may be obtained. An investigation of this has been carried out by Richards, McCaffrey and Bisbee [1901], who show that the quantity of magnesium oxalate occluded by calcium oxalate depends on the concentration of undissociated magnesium oxalate. This quantity is decreased by the addition of ammonium chloride, which forms complex compounds with magnesium. Ammonium chloride has a similar solvent effect, but to a much smaller extent, on calcium oxalate.

An excess of ammonium oxalate would increase the quantity of undissociated magnesium oxalate and this is avoided by adding the reagent slowly, an excess of ammonium oxalate being added only after precipitation has taken place.

The most favourable conditions are obtained when an acid solution is slowly neutralised by ammonia, and an excess of ammonium oxalate is added afterwards.

In the following determinations, in which due regard was paid to these conditions, which were established by Richards and his associates, the following method was adopted for determination in serum-ash and in stock solution.

0·02 cc. tropaeolin 000 No. 1 is added to 1 cc. solution in a glass tube such as is described in the paper of Trevan and Bainbridge, and the liquid is stirred with a small glass rod. 0·01 cc. 2·5 % ammonium chloride, 0·8 cc. $N/100$ oxalic acid, and 0·12 cc. $N/10$ hydrochloric acid are then added successively. The liquid is then neutralised by the addition of 0·2 N ammonia, 0·01 cc. being added at a time, with a 5 minutes' pause after each addition. The neutralisation is continued until the colour indicates a p_H of 8·0, a buffer solution being used for comparison. A further 0·6 cc. of 3·5 % ammonium oxalate is then added, and the mixture is put aside for four hours to precipitate, the further treatment being as indicated by Trevan and Bainbridge. This method of neutralising differs somewhat from that of Richards who used methyl orange as an indicator, heating the liquid to boiling point, this being necessary when the calcium oxalate is to be filtered off. The precipitate is collected in this case by centrifuging, and may be formed without heating. There was no difficulty about the addition of alkali to p_H 8·0, and the colour changed back again towards the acid shade when the excess of ammonium oxalate was added.

Richards advises the addition, when calcium has to be determined, of an amount of ammonium chloride ten times as large as the amount of magnesium present. In the analyses of which the results are here published, the quantity of ammonium chloride added was only roughly equivalent to the magnesium, a considerable quantity of ammonium chloride being formed during neutralisation, and the metals being present in a very dilute solution.

By taking all these precautions, Richards, McCaffrey and Bisbee obtained results which were approximately equal to the right values. These were supposed to be due to the fact that two slight errors neutralised each other, viz. a small loss of calcium, and the occlusion of some magnesium in the precipitate. Phosphates are not mentioned in their paper.

The method of Kramer and Tisdall [1921], which was afterwards simplified by Tisdall [1923], has furnished some data on this point with regard to serum. The original method of Kramer and Tisdall was based on the fact that in the presence of phosphates calcium oxalate can be precipitated in acetic acid solution.

The method of McCrudden [1909–10] for a quantitative separation of calcium, magnesium, and phosphates in food, urine, and faeces was modified for

that purpose. Kramer and Tisdall precipitated the calcium oxalate in serum or plasma after adding acetic acid. But Tisdall found later that when nothing was added to serum but ammonium oxalate, the precipitate of calcium oxalate was sufficiently pure, and his simplified method of precipitation is about the same as that already applied by de Waard [1919]. In mixtures dissolved in water, which were approximately neutral, McCrudden obtained a precipitate which contained calcium phosphate. This, however, took place in rather different conditions from those of the method here employed for solutions of serum-ash and analogous stock solutions.

Tests were made with the precipitate obtained by the latter method for phosphates. A liquid was used as a stock solution, the composition of which was about the same as one of the ash of serum, containing per 100 cc.:

21·8 mg. K_2SO_4.
22·9 mg. Na_2HPO_4.
39·1 mg. KCl.
800·0 mg. NaCl.
25·0 mg. $CaCO_3$ (dissolved in HCl).
21·0 mg. $MgCO_3$ (dissolved in HCl).

As much hydrochloric acid was added as was required to dissolve the whole of these substances.

The calcium oxalate was slowly precipitated in 10 cc. of this stock solution. Calcium oxalate was also obtained from 10 cc. of serum by simply following the method of Trevan and Bainbridge. To prevent the chlorides from upsetting the test for phosphates the precipitate was washed as usual, and then dried after adding nitric acid. The solution was allowed to stand for 4 hours at 50° mixed with ammonium molybdate reagent. The same was done with 1 cc. 0·0045 % Na_2HPO_4, $2H_2O$. The amount of phosphate present in 10 cc. of the stock solution was thus 60 times as great as in 1 cc. of the phosphate solution. This latter gave a distinctly positive reaction. Both the others, on the contrary, yielded a practically inappreciable amount of yellow precipitate.

The object of the next experiments was to find out what calcium values are found by the Trevan-Bainbridge method.

In a stock solution containing nothing but 8 mg. of calcium and 6 mg. of magnesium per 100 cc. of the liquid, the calcium was precipitated by that method, that is, without neutralising slowly. The values obtained (mg.) in three series were:

I. 8·4, 8·4, 8·0, 8·4, 8·2, 9·0.
II. 8·6, 8·6, 9·0.
III. 8·4, 8·6, 8·2, 8·8.

When slow precipitation was applied, four series showed:

I. 8·2, 8·0, 8·0.
II. 8·3, 8·1, 8·4.
III. 7·6, 7·6, 7·8, 7·6.
IV. 8·2, 8·0, 7·8, 8·0.

A stock solution containing 10 mg. of calcium and 6 mg. of magnesium in 100 cc., direct precipitation being applied, yielded:

11·0, 10·7, 10·8, 10·7, 10·4.

Slowly precipitated:

9·6, 9·8, 9·7, 9·6, 9·7, 10·5.

When the average values of these series are considered, it is evident that a small percentage of error in either sense was not avoided. The higher values obtained by direct precipitation are demonstrated by these tests.

It was also found possible to perform a slow neutralisation in serum. But no difference in the values was found between the results and those of the direct precipitation by the Trevan-Bainbridge method. The values given are the average of several determinations:

	Direct precipitation	Slow precipitation
First serum	9·3	9·3
Second serum	10·4	10·4
Third serum	10·2	10·4

The higher values, attributed to the occlusion of magnesium, do not appear in this case. A test for magnesium was made in the precipitates by the reaction of Hahn, Wolf and Jäger [1924]. This showed that, when the calcium oxalate was precipitated directly, the test for magnesium was distinctly positive, but when it was precipitated slowly, the reaction was less intense, but not quite negative. In serum the calcium oxalate seems to be precipitated more slowly, and the occlusion of magnesium is apparently less.

The question is now whether more calcium can be obtained by allowing the mixture to stand for a longer time, or by adding more highly concentrated ammonium oxalate.

Calcium was now precipitated from ox-serum, but in this case by the addition of 2 cc. of 7 % ammonium oxalate, which was kept in solution by means of gentle warmth. For the rest, the method of Trevan and Bainbridge was followed, and in addition to this the usual precipitation was performed with 3·5 % ammonium oxalate. The former determinations gave 12·0 mg. of calcium, the latter 10·1 mg. in 100 cc.

The experiment was repeated with human serum; 7 % ammonium oxalate gave a value of 10·6 mg. of calcium, and 3·5 % ammonium oxalate gave 9·9 mg. The tests for magnesium again differed, being more positive when 7 % ammonium oxalate was used, but not quite negative with 3·5 % ammonium oxalate.

The method of Trevan and Bainbridge was then again employed, with a different time for precipitation. The test was made with sheep-serum. After standing 1 hour for precipitation 9·3 mg. Ca were found, after 4 hours 9·3 mg. Ca, and after 24 hours 9·2 mg. Ca per 100 cc. The test for magnesium was slightly positive in all three cases, but no difference in quantity was visible. A small amount of magnesium seems always to be occluded, but when the method of Trevan and Bainbridge is followed, the amount remains within the limits of the error which, with the tests here made, was unavoidable.

The following data may serve as an example of the degree of accuracy attained:

Solution used	mg. Ca per 100 cc. found	Average
Stock containing 8 mg. Ca per 100 cc.	8·4, 8·3, 7·6, 8·2, 7·7	8·0
Stock containing 10 mg. Ca per 100 cc.	10·1, 10·4, 10·1, 10·1, 10·3	10·2
Stock resembling serum-ash and containing 10 mg. Ca per 100 cc. Neutralised slowly	9·8, 10·2, 10·2, 10·6	10·2
	9·6, 10·1, 10·1, 10·2, 10·1, 10·1	10·0
	9·7, 10·1, 9·7, 9·7	9·8
Normal serum I	10·5, 10·2, 9·9, 10·2, 10·3	10·2
Normal serum II	10·5, 10·5, 10·4, 10·2	10·4
Normal serum III	10·0, 10·2, 9·8, 10·2	10·05

Tests in ash and serum were now made side by side—in the ash solution by slow neutralisation.

The ashing was done by the method of Stolte [1911]. 5 cc. of serum were ashed in a platinum crucible, which was placed in a quartz crucible and covered with a quartz cover. The ash obtained was practically white, and most of it could be transferred to a centrifuge tube marked at 5 cc. The rest of the ash was dissolved in 1 cc. of N HCl, and this liquid was introduced into the tube, the volume being brought up to 5 cc. by washing with water. After centrifuging, a clear liquid was obtained, of which 1 cc. at a time was transferred to tubes for Trevan and Bainbridge's calcium test. The acid solutions, on being heated to dryness, left a crystalline residue, in which, after it had been dissolved in 1 cc. of water, the calcium oxalate was precipitated by slow neutralisation. Most of the tests were done in ox-sera, some of which were haemolytic.

Serum	Ash
10·9	10·7
11·4	11·1
9·2	9·4
10·7	10·7
10·9	11·3
9·9	10·0

Occasionally a test was made in a stock solution by way of a check. Tests were also made on the sera of ten persons in whom a normal calcium value might be expected. The averages of these determinations were:

10·3	10·3	9·8	10·4	10·2
10·0	10·2	9·9	10·0	10·3

Finally, some determinations by the method of Trevan and Bainbridge were compared with the values given by two permanganate methods. It may be remarked that by the latter methods, which require to be carried out with the utmost regularity, more constant values would probably be found in the long run.

Most of the values simultaneously obtained for one serum did, however, correspond fairly well. For instance:

	Method of Clark and Collip [1925]	Method of de Waard [1919]
Serum IV	10·8	9·8
	10·5	9·8
	10·8	10·2
	10·5	10·2
	10·7	
Serum VI	9·9	10·0
	9·8	10·0
	10·0	

The average results of the three methods were:

	Trevan and Bainbridge	Clark and Collip	de Waard	Ash
I	11·4	11·6	11·1	11·1
II	11·3	11·3	11·0	—
III	9·2	9·6	8·8	9·4
IV	10·7	10·7	10·0	10·7
V	10·9	11·8	11·3	11·3
VI	10·2	9·9	10·0	—
VII	9·8	10·2	10·5	—
VIII	10·4	10·5	10·2	—

A check seems also advisable with permanganate methods, if the tests are not carried out by the same person. The method of Trevan and Bainbridge occasionally gave distinctly wrong results, as shown by the considerable divergence in one series of values. They were evidently caused by heating too much or too little.

Summary.

When calcium oxalate is slowly precipitated, so as to avoid the occlusion of magnesium, values which agree fairly well are obtained by the determination of calcium in serum and ash according to the Trevan-Bainbridge method. The degree of accuracy obtained by Trevan and Bainbridge in their tests was, however, not attained here. The conditions for the precipitation of calcium are, so far as the influence of magnesium is concerned, evidently favourable in serum.

As a standard solution for comparative tests a solution may very well be taken which contains calcium only, in which case the method of Trevan and Bainbridge can be followed in its entirety.

The results given by the Trevan-Bainbridge method were, in the case of a limited number of determinations, compared with those of two permanganate methods. The method of Clark and Collip generally gave somewhat higher, that of de Waard generally somewhat lower values than the Trevan-Bainbridge method.

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CXXXIV. BLOOD-FAT.

II. THE ACETONE-ETHER-SOLUBLE FRACTION.

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(*Received October 31st, 1929.*)

In a previous paper [Channon and Collinson, 1929] a method of preparing blood-fat has been described and the general nature of a number of samples has been discussed. In the large-scale preparation, 40 l. of ox blood at fasting level yielded 158·9 g. of fat, which contained 1·57 % P, 2·39 % N, 17·04 % sterol precipitated by digitonin, 6·43 % sterol present in ester form, and 8·24 % unsaponifiable matter other than sterol; it yielded 32·46 % of its weight as fatty acids which had molecular weight 294 and iodine value 100.

136 g. of the fat were dissolved in 400 cc. ether and the warm solution was poured into 1·8 l. acetone with vigorous shaking, after which the mixture was maintained at 0° for 7 days. The precipitated phosphatide fraction was removed by filtration, ground in a mortar with acetone and the mixture filtered. This operation was repeated three times, and the acetone washings were united to the ether-acetone mother liquor. The acetone-insoluble fraction was then shaken with ether, when a portion of it was found to be insoluble. After standing, the ether solution was siphoned off, and the residue again treated with ether three times, and the ether washings combined. In this way, the 136 g. of fat were resolved into three fractions: (*a*) soluble in acetone-ether, 47 g.; (*b*) precipitated from ether solution by excess of acetone and re-dissolved in ether, 69·6 g.; (*c*) precipitated from ether solution by excess of acetone, but found to be no longer soluble in ether, 10 g.

The present communication discusses the nature of fraction (*a*), the acetone-ether-soluble fraction. It was an orange mass which contained 0·87 % N and 0·05 % P. About 87 % of the nitrogen present in the original material was precipitated in the phosphatide fractions (*b*) and (*c*), while the phosphorus content of the fraction corresponds to about 1·3 % of lecithin. The material was stored in the dark in a flask completely filled with ether for 12 months before opportunity to investigate it arose.

Experimental.

A small amount of precipitate which had formed on standing was removed by filtration[1]. The remaining material was found by saponification to contain 70·13 % of unsaponifiable matter, 91·81 % of which was cholesterol, and 27·28 % of fatty acids. There were thus present in the material (45·53 g.), 29·31 g. of free and combined cholesterol, 2·63 g. of unsaponifiable matter other than sterol[2] and 12·43 g. of fatty acids.

The material contains free cholesterol and cholesterol esters together with glycerides, and possibly traces of free fatty acids and soaps.

The object of the subsequent work was to attempt to gain some idea as to what fatty acids were in combination with cholesterol and to what extent the fraction was composed of glycerides. On account of the similar solubilities of cholesterol and its esters in the usual fat solvents, the separation by fractional crystallisation of small amounts of cholesteryl esters in any state of purity from a mixture containing a high percentage of free cholesterol is a matter requiring large amounts of material, and even then the complete identification of the esters is difficult unless they be obtained in quantity. Hence the nature of the constituents of the ester mixtures obtained by Hürthle [1895] and Bloor [1924] remains uncertain. In view of these difficulties, therefore, it was considered advisable to attempt the separation of the material into two fractions, (*a*) cholesterol and cholesteryl esters, and (*b*) glycerides, instead of attempting to isolate the individual cholesteryl esters present. If such a separation, based on the fact that cholesteryl esters have a lower solubility in fat solvents than glycerides, were possible, the identification of the fatty acids produced by the hydrolysis of fraction (*a*) would indicate what cholesteryl esters were present. The material was dissolved in 400 cc. acetone and on cooling yielded 20·25 g. of crystalline solid (fraction 1). Further crystallisation was carried out and four fractions, two of which were oils, were obtained, together with 9·06 g. of sticky solid obtained from the mother liquor (fraction 3). The amount of free cholesterol in fraction 1 was determined, and the remainder was saponified and the weight of unsaponifiable matter and fatty acids obtained. The results of these estimations showed that the amount of fatty acid was greater than that required by the esterified cholesterol present, and demonstrated that the fractional crystallisation was unsuccessful. Accordingly, the four intermediate fractions were united to form fraction 2.

[1] After repeated boiling with charcoal and crystallisation from acetone, a mixture of needles and an amorphous substance was obtained. This mixture, 0·2 g., contained 35·08 % N.

[2] The analytical figures determined on the original blood-fat show that there should be present 31·9 g. of total cholesterol and 11·20 g. of unsaponifiable material other than cholesterol. There thus appears to have been a loss of 2·59 g. of cholesterol and 8·57 g. of unsaponifiable material other than cholesterol. The former loss is attributable to the manipulations involved. The apparent loss of about 80 % of the unsaponifiable material other than cholesterol may be due either to its being precipitated together with the phosphatides or alternatively to its production by the materials used in the alkaline saponification. The latter view is almost certainly correct.

Quantities of about 1 g. of fractions 2 and 3 were set aside for the purpose of estimating glycerol. After determination of the amount of free cholesterol, the remainder of each fraction was then resolved after saponification with sodium ethoxide into its constituent unsaponifiable matter and fatty acids. These results together with those obtained from the whole of fraction 1 are recorded in Table I, together with the iodine values of the fatty acids.

Table I.

Fraction	Weight (g.)	Unsap. matter (%)	Free sterol (%)	Fatty acids (%)	Iodine value of fatty acids
1	20·24	78·3	56·9	20·11	110·1
2	9·18	69·2	37·3	27·39	137·9
3	10·17	49·5	22·2	38·80	122·6

In Table II are recorded the weights of the components corresponding to the percentages recorded in Table I. The percentage of total cholesterol present in each unsaponifiable fraction was not estimated, because, even if the unsaponifiable fractions had been wholly cholesterol, there would still be an excess of fatty acid in each fraction over that required by the cholesteryl ester present. It has been mentioned already that the unsaponifiable fraction determined on an aliquot part of the whole material prior to resolution into three fractions contained 91·81 % of cholesterol, and accordingly this figure has been used for the calculations of the total sterol in each unsaponifiable fraction. As the mean molecular weight of the fatty acids in these three fractions was 285·2, the weight of fatty acid necessary for combination with the amount of cholesteryl ester present has been calculated as stearic acid (M.W. 284). The amount of fatty acid in excess of that required by the cholesteryl ester is recorded in the last column. The weights of the fractions are the original weights prior to removal of the aliquot parts used for other determinations.

Table II.

Fraction	g.	Unsap. matter (g.)	Total sterol (g.)	Free sterol (g.)	Sterol ester (g.)	Fatty acid equivalent of sterol ester (g.)	Fatty acids found (g.)	Fatty acids present in other forms (glyceride)
1	20·24	15·85	14·55	11·52	3·03	2·23	4·07	1·84
2	9·18	6·35	5·83	3·43	2·40	1·77	2·51	0·74
3	10·17	5·03	4·62	2·26	2·36	1·74	3·95	2·21
Total	39·59	27·23	25·00	17·21	7·79	5·74	10·53	4·79

From these results it is clear that the 39·59 g. of material contains no more than 4·79 g. of fatty acid in excess of that required for the cholesteryl esters present. If allowance be made for the 4·53 g. withdrawn from the main bulk before fractional crystallisation, this amount of fatty acid becomes 5·34 g. Further, if the phosphorus content of the original 45·53 g. (0·05 % P) be due to the presence of lecithin, the latter would give rise to 0·4 g. of fatty acid on hydrolysis. Hence the amount of fatty acid obtained by hydrolysis of the ether-acetone fraction which is not in combination with cholesterol is 4·94 g.

from 34·2 l. of blood, or about 15 mg./100 cc. blood. Although, as already pointed out [Channon and Collinson, 1929], the method used for extracting the blood has resulted in not more than 80 % of the fat being extracted, it seems probable that the figure of 15 mg./100 cc. is a maximum, for it is to be anticipated that what little glyceride is present in blood is readily extracted, whereas the phosphatide and cholesteryl esters will be removed completely only with difficulty.

The fatty acids.

The remainder of the fatty acids, 9·58 g., were separated into a solid fraction, 2·04 g., and a liquid fraction 6·06 g., by the lead salt and ether process. These two fractions had iodine values of 30·1 and 150·8 respectively.

The solid fraction. After boiling the acetone solution of the saturated acids with charcoal, two fractions (1) 0·23 g., (2) 0·86 g. were obtained. These analysed as follows.

Fraction (1) 0·1366 g. substance required for neutralisation 5·0 cc. 0·1 *N* KOH.
4·920 mg. substance: 13·65 mg. CO_2 and 5·68 mg. H_2O.

Fraction (2) 0·1127 g. substance required for neutralisation 4·22 cc. 0·1 *N* KOH.
4·400 mg. substance: 12·08 mg. CO_2 and 4·96 mg. H_2O.

	C	H	M.W.	M.P.
Calculated for $C_{16}H_{32}O_2$:	75·01	12·50	256	63–64°.
$C_{18}H_{36}O_2$:	76·07	12·68	284	70–71°.
Found, fraction (1)	75·65	12·82	273	57·5–58°.
(2)	74·85	12·52	267	54–55°.

The amount of material available was obviously too small for the definite identification of the acids present, but the saturated acids appear to be a mixture of stearic and palmitic acids.

The liquid fraction. 5·8 g. were brominated in anhydrous ether at 0°. After standing overnight, the precipitate 0·6 g. (fraction 1) was filtered off, and washed with ether. The excess bromine was removed from the mother liquor by shaking with sodium thiosulphate solution. The ether solution after drying with anhydrous sodium sulphate was concentrated to 25 cc. and cooled to 0° when a further precipitate (0·2 g.) was removed. Excess of light petroleum was added and the ether removed by evaporation until the solution was turbid at room temperature. The solution was cooled to 0° overnight and 1·3 g. of precipitate (fraction 2) were removed. After a further 5 days at 0° a second precipitate 0·4 g. (fraction 3) was obtained. The mother liquor from fraction 3 was completely soluble in light petroleum. The fractions analysed as follows.

Fraction (1) 4·73 mg. substance: 4·58 mg. CO_2 and 1·74 mg. H_2O.
0·1067 g. substance required (Stepanoff) 8·9 cc. 0·1 *N* thiocyanate.

Fraction (2) 4·582 mg. substance: 5·710 mg. CO_2 and 2·09 mg. H_2O.
0·0753 g. substance required 5·23 cc. 0·1 *N* thiocyanate.

Fraction (3) 4·841 mg. substance: 6·420 mg. CO_2 and 2·58 mg. H_2O.
0·0971 g. substance required 6·49 cc. 0·1 *N* thiocyanate.

Mother liquor 0·1488 g. substance required 8·0 cc. 0·1 *N* thiocyanate.

	C	H	Br	M.P.
Calculated for $C_{20}H_{32}O_2Br_8$:	25·42	3·39	67·79	(decomp.) 240°.
$C_{18}H_{30}O_2Br_6$:	28·49	3·99	63·26	181°.
$C_{18}H_{32}O_2Br_4$:	36·00	5·33	53·28	114°.
$C_{18}H_{34}O_2Br_2$:	48·88	7·69	36·2.	
Found, fraction (1)	26·4	4·09	66·71	(decomp.) 238°.
(2)	33·98	5·07	55·60	105–107°.
(3)	36·16	5·92	53·48	114°
Mother liquor			43·0	

The analytical figures for fraction 1 approximate to those required by arachidonic acid octabromide. Fraction 2 appears to consist of a mixture of tetrabromostearic acid and of the bromides of linolenic acid or arachidonic acid. Fraction 3 is tetrabromostearic acid. The mother liquor yielded a syrup containing 43·0 % bromine intermediate between the values required by the tetrabromide (53·28 %) and the dibromide (36·2 %). The finding of arachidonic acid in the acetone-soluble fraction is of interest since it was shown to be present in liver-lecithin by Levene and Simms [1922]. Since the small amount of phosphatide present in the fraction would give rise to 0·2 g. of unsaturated acids, it is just possible that the amount found could have been derived from the phosphatide present. This, however, seems improbable, since the phosphatides isolated from varying sources give rise on hydrolysis to mixtures of unsaturated acids.

Discussion.

The source of the residual fatty acids. During the subsequent discussion, the fatty acids which are present in the blood in forms other than as cholesteryl esters or as phosphatide will be referred to as the residual fatty acids, for the sake of ease of description. Information as to whether these residual fatty acids are present in the blood in the post-absorptive state as neutral fat, free fatty acid or possibly as soap is almost entirely lacking. It is of interest to note, however, that Letsche [1907] concluded that if glycerides were present, they constituted an insignificant portion of the fat. Since it was not possible to separate the glycerides from the cholesterol and cholesteryl esters present, estimations of the glycerol content were carried out on quantities of about 1 g. of fractions 2 and 3 by the method of Zeisel and Fanto [1903]. The results showed that these fractions contained glycerol corresponding respectively to 4·69 % and 9·59 % of neutral fat calculated as tristearin, or 0·43 and 0·97 g. These quantities account for 56 % of the residual fatty acids in fraction 2 and 42 % in the case of fraction 3. A determination was not carried out on fraction 1 as material was not available, but the results on fractions 2 and 3 suggest that about 50 % of the residual fatty acids are coupled to glycerol as neutral fat, *i.e.* the blood contains 7–8 mg. of neutral fat per 100 cc. Whether the remainder is present as free fatty acids or soaps,

we have no information, but the method of Zeisel and Fanto applied to materials containing so small a percentage of glyceride will tend to give low values. Jarisch [1923] concluded, from a study of the behaviour of soaps in solutions of buffers at varying p_H values, that the reaction of the blood is such as to preclude the presence of soaps.

The amount of the residual fatty acids in blood. In view of the dearth of information regarding the analysis of blood-fat on a large scale, it is of interest to compare our findings with those obtained by micro-methods. For the determination of the various fatty compounds in blood, two methods, differing both in the technique employed for the extraction of the blood and the subsequent treatment of the extracts obtained, have been worked out by Bloor [1915] and by Bang [1918, 1]. Bloor's method entails nephelometric determinations of total fatty acids and ether-soluble phosphorus on an alcohol-ether extract of blood, together with a colorimetric determination of cholesterol. The amount of the residual fatty acids may then be calculated by subtracting the sum of those present as phosphatide (obtained from the ether-soluble phosphorus figure) and of those present as cholesteryl esters from the total fatty acids. Bang's method depends on the extraction of air-dried blood with light petroleum followed by alcohol. The former solvent is reported by Bang [1918, 1] and by Blix [1925, 1, 2] to remove free cholesterol and neutral fat only, and the latter, cholesteryl ester and phosphatide. The amount of the components present in the two extracts is estimated after suitable treatment by oxidation under prescribed conditions with potassium dichromate. Hence Bang's method entails a direct estimation of the glyceride content, while Bloor's figures are obtained indirectly. Some of the results recorded in the literature are presented in Table III.

Table III. *Amount and distribution of fatty acids of blood expressed as mg./100 cc.*

Animal	Mean of	Method	Total	As lecithin	As cholesteryl ester	Residual (glyceride)	Observer
Dog	10	Bloor [1915]	552	238	34*	280	Bloor [1915]
	7	,,	594	243	44*	307	Bloor [1916, 1]
	6	,,	713	208	40	465	Knudson [1917]
	12	Bang [1918, 1]	276	181	66	29	Bang [1918, 2]
Man	14	Bloor [1915]	360	210	52†	98	Bloor [1916, 2]
Woman	9	,,	360	203	57†	100	,,
Man	7	Bang [1918, 1]	225	131	70	24	Bang [1918, 2]
	15	,,	—	—	—	20	Blix [1925, 2]
Woman	21	,,	—	—	—	30	,,

The figures recorded for the fatty acids present as phosphatide and cholesteryl ester have been calculated by the writers from those in the papers referred to (fatty acids = 0·70 × lecithin, fatty acids as cholesteryl ester = ester cholesterol × $\frac{9 \cdot 5 \cdot 5}{3 \cdot 1 \cdot 4}$).

* Total cholesterol only was determined. This figure has been calculated from the results of Knudson [1917] who found 26·4 % of the total sterol in dog's blood to be in ester form.

† Calculated similarly from the results of Bloor and Knudson [1917] who found that 34 % of the total cholesterol present in human blood was in ester form.

The results recorded in Table III show that the figures yielded by Bloor's method are consistently greater than those obtained by that of Bang. The

differences in the figures appear to be much in excess of what might be expected to be due to normal variation in the amount of blood-fat at fasting level, for each is a mean value derived from determinations on the blood of six or more animals. The main difference appears in the residual fatty acids, for those obtained by Bloor's method are from 6 to 15 times as great as those recorded by Bang for dog's blood, and from 3 to 5 times as great in the case of human blood. Since from the study of blood-fat in bulk we have arrived at a figure of 15 mg. per 100 cc. for the residual fatty acids of ox blood, a figure which is of the same order as those reported by Bang and Blix for blood from a large number of dogs, men and women (20–30 mg./100 cc.), our results, as do those of Letsche [1907], may suggest that Bang's method yields results for the residual fatty acids more reliable than that of Bloor. It may perhaps be unsafe to compare the results obtained from the blood of a herbivorous animal with those from that of carnivorous animals. Horiuchi [1920], however, using Bloor's method on the blood of rabbits, obtained results which suggested that 40–50 % of the fatty acids were present as residual fatty acids, figures of the same order as are recorded in Table III for the blood of dog and man by this method. The possibility exists, however, that figures for the residual fatty acids, even though they be obtained from blood in the post-absorptive stage, may vary considerably among themselves on account of the state of previous nutrition of the animal. The finding of relatively small amounts of fatty acid not in combination with cholesterol or present as phosphatide bears out the results previously recorded on twelve samples of blood [Channon and Collinson, 1929].

The degree of unsaturation of the fatty acids of the phosphatide and acetone-soluble fraction. During the investigations of the nature of the fatty acids of the phosphatide fraction, it became apparent that the phosphatide fatty acids were definitely more saturated than those of the acetone-soluble fraction. Further samples of blood-fat were prepared and were approximately separated by the addition of excess of acetone to their solution in ether into phosphatide and acetone-soluble fractions: without further purification the fractions were saponified and, after removal of the unsaponifiable matter, the fatty acids were obtained and their iodine values were determined. For comparison with the figures so obtained, there are recorded in Table IV the iodine values of the corresponding fractions of the liver, heart and kidney of rats, which had received for some weeks a synthetic diet in which butter was the source of fat. These figures on rat tissues are recorded instead of figures on the organs of the ox, because they are representative average values, since the two groups (*a*) and (*b*) contained 25 and 17 animals respectively.

Bloor [1927, 1928] obtained for the iodine values of the fatty acids of the phosphatide fractions of the liver, kidney and heart of the ox 113, 110 and 120, and for those from the acetone-soluble fraction 87, 82 and 75 respectively. It is clear from the data recorded in Table IV, firstly, that the phosphatide fatty acids of blood are considerably less unsaturated than those of the tissues, and

Table IV. *Iodine values of the fatty acids of the phosphatide and acetone-soluble fractions of the fat of ox blood at fasting level.*

		Phosphatide fatty acids		Fatty acids of acetone-soluble fraction	
Ox	Blood	71 77 86 90		123 137 143 121	
		(*a*)	(*b*)	(*a*)	(*b*)
Rat	Liver	153	149	76	80
	Kidney	129	113	64	66
	Heart	133	128	83	75

secondly, that the fatty acids in the acetone-soluble fraction are considerably more unsaturated than those of the phosphatide fraction. These figures are of the same order as those of Bloor [1924] who found that the fatty acids of the phosphatide fraction of ox plasma had an average iodine value of 71 determined on 16 samples, whereas the average iodine values of the fatty acids of the successive fractions obtained by crystallisation of the acetone-soluble fraction from alcohol were 139, 147, 111, 119 and 94.

Before discussing the possible physiological significance of the degree of unsaturation of the fatty acids of the phosphatide and acetone-soluble fractions, the evidence as to whether the unsaturated acids in the acetone-soluble fraction are present as glyceride or as cholesteryl ester will be considered. Meanwhile, it may be noted here that, as in blood, the fatty acids of yeast-phosphatides are more saturated than those of the acetone-soluble fraction [Daubney and Smedley-MacLean, 1927], and the same finding holds for the corresponding acids from cabbage leaf cytoplasm [Chibnall and Channon, 1929].

The compounds in which the unsaturated acids are present. Earlier in this paper, it was shown that about 55 % of the fatty acids of the acetone-soluble fraction are coupled to cholesterol as cholesteryl esters, and that the remaining 45 % are present probably chiefly as glyceride. The amounts of the fatty acid obtained from the fractions 1, 2 and 3 were 82, 42 and 127 % in excess of that required by the cholesteryl ester present, and the iodine values of the mixed fatty acids from the three fractions were 110, 138 and 123 respectively. It was shown also that the combined fatty acids of the three fractions contained arachidonic, linoleic and oleic acids. Since the fatty acids obtained by hydrolysis of each fraction contain so large an amount of residual acid, no reliable deduction can be drawn from consideration of their iodine values as to the degree of unsaturation of that fraction of the acids which was coupled to cholesterol. Thus, if those acids not present as cholesteryl esters were arachidonic and linoleic acids, the acids coupled to cholesterol would have a relatively low iodine value. Hence analysis of our own results leaves us unprepared to express a definite view on this point. On the other hand, Bloor [1924], from a study of the proportions of unsaponifiable matter and fatty acid produced by hydrolysis of the first two fractions obtained by crystallisation from alcohol of the acetone-soluble fraction of 16 samples of plasma-fat,

concludes that these fractions consist of cholesteryl esters and deduces from the iodine values of the fatty acids that the unsaturated acids of plasma are mainly present as esters of cholesterol. Since Bloor's calculated figures for the fatty acids are obtained by regarding the unsaponifiable matter as entirely cholesterol, the agreement with the amounts of fatty acid found is more apparent than real, for it is stated later that the unsaponifiable matter consisted of from 70 to 90 % of sterol only. Hence, disregarding the fact that any free cholesterol in the fractions will lower still further the theoretical amount of fatty acid required, analysis of Bloor's figures shows that if the sterol content of the unsaponifiable fractions be taken as 80 % on the average, the fatty acids obtained by hydrolysis were in excess of the theoretical amount in 11 cases in fraction 1 from 21 to 54 % and in fraction 2 from 23 to 40 %. Hence for the reasons mentioned in the case of our own results, no conclusion can be safely drawn as to what proportion of the unsaturated acids is present as cholesteryl esters.

The transport of unsaturated acids from the liver to the tissues. On the evidence of Leathes and Meyer-Wedell [1909] and Hartley [1909], Leathes and Raper [1925] consider that one function of the liver is to desaturate fatty acids in preparation for their utilisation by the tissues. Hence, evidence regarding the unsaturation of the fatty acids in the various fat compounds in blood should afford some evidence as to the form in which these desaturated fatty acids are carried to the tissues from the liver.

Since the fatty acids of the acetone-soluble fraction of blood-fat are considerably more unsaturated than those of the phosphatide, it might seem reasonable to suggest that the unsaturated acids are carried to the tissues in this fraction.

On the other hand, the fact that the phosphatide fatty acids of blood are more saturated cannot be taken as evidence that the phosphatides are not playing a part in fat transport. If the researches of Levene and his colleagues on the phosphatides of tissues, which have shown that the fatty acids of these compounds are made up of equimolecular proportions of saturated and unsaturated acids, apply to the blood-phosphatides, then the iodine value of the unsaturated half of the mixed fatty acids, which have an iodine value of 80, will be 160. If these unsaturated acids contain oleic acid, the iodine value is sufficiently high for there to be present in the phosphatide molecule acids considerably more unsaturated than oleic. Hence the relatively low iodine value of the blood-phosphatide acids does not preclude the phosphatides from being considered as carriers of unsaturated acids from the liver to the tissues.

SUMMARY.

1. The acetone-soluble fraction of the fat from 34 l. of ox blood at fasting level has been investigated.

2. The fatty acids present in the fraction are arachidonic, linoleic, oleic, stearic and palmitic acids.

3. Of the fatty acids produced by hydrolysis of the fraction, 55 % are present as cholesteryl ester. Of the remainder, about one-half is present as neutral fat.

4. The amount of fatty acid in the fraction which is not in combination with cholesterol corresponds to no more than 15 mg./100 cc. blood. The bearing of this finding on certain micro-methods of blood-fat determination is discussed.

5. The acids obtained by hydrolysis of the fraction are considerably more unsaturated than those of the phosphatide fraction; but whether the unsaturation is mainly due to the fatty acids present as glyceride or as cholesteryl ester has not been determined.

6. The bearing of the results on the question of the transport of unsaturated acids from the liver to the tissues is discussed.

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CXXXV. A NOTE ON SELACHIAN YOLK-PROTEINS.

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(*Received November 6th, 1929.*)

Considerable interest attaches to the proteins of egg-yolk throughout the animal kingdom, but, although those which occur in avian yolk have been fairly well investigated, little has been done with those of the eggs of other groups. As a result of the researches of Bayliss and Plimmer [1906], Plimmer and Scott [1908], Kay and Marshall [1928] and others, we know that only two proteins exist in the egg-yolk of the hen, vitellin, a phosphoprotein, and livetin, a pseudoglobulin, the latter being identical with the so-called Gross's protein [Gross, 1899]. As regards other eggs, however, our information is fragmentary, for while a substance called icthulin, very similar in its properties and constitution to vitellin, has frequently been found in fish eggs, we do not know whether it is the only protein there, or whether it exists in the eggs of such groups as insects and crustacea.

A supply of the egg-yolk of the selachian *Acanthias vulgaris* (the "spur-dog") being available owing to the necessities of other work, it was thought worth while to investigate two points concerning its proteins; (*a*) the presence of phosphorus in the selachian icthulin, and (*b*) the presence of any other protein in the yolk. The first of these arises out of the work of Alsberg and Clark [1908] which forms the only other study of the eggs of this fish. Alsberg and Clark prepared the icthulin following the classical method of solution in saline and precipitation by dilution, and then, determining the elementary composition of their preparation, found to their surprise that it contained no phosphorus. The eggs of *Acanthias vulgaris*, they concluded, contain no vitellin in the strict sense of the word, but in its place a globulin or a mixture of globulins containing no phosphorus and perhaps no iron. They also put forward the suggestion that this state of affairs, which formed such a striking exception to all other vertebrate eggs, might be connected with the fact that the fish is ovoviviparous.

In the present work the yolk (which is contained within a vitelline membrane forming a sphere about 3 cm. in diameter and flows out as a pale yellow creamy material thinner than avian yolk) was thoroughly mixed with an equal volume of 10 % NaCl and brought to the laboratory in that form. The yolks from 16 eggs were used, *i.e.* 368 g. of raw material. The mixture was now extracted

with ether in a separating funnel until no more pigment and fat passed into the solvent, and then precipitated by dilution with distilled water. When the precipitate had settled it was centrifuged off and again dissolved in 10 % salt solution to be again precipitated by dilution with water, the supernatant liquid being retained for further examination. After five precipitations in this way, the protein was washed with ether-water by successive decantations until no more chloride could be found in the washings, during which time it was observed that the protein settled less and less readily. Then it was transferred to a flask and boiled out with alcohol for 5 days under a reflux condenser, the alcohol being frequently changed. Finally, the material was dried for 6 months *in vacuo* over sulphuric acid.

The protein so obtained was a greyish powder which gave positive results with the biuret, glyoxylic, and Millon reactions. Its solubilities were such as are usually found with vitellins, but no work was done on its coagulation temperature or other physical properties. In Table I are shown the data for elementary composition as determined by Alsberg and Clark and in the present work. The main difference consists in the results for phosphorus, which was determined after a thorough incineration by the colorimetric method of Fiske and Subbarow [1925]. No difficulty was experienced in showing its presence in about the same percentage as is usually found in vitellins and it is therefore difficult to explain their failure to identify it.

Table I.

	C %	H %	N %	P %	S %	
Selachian egg-yolk						
Icthulin	50·49	7·16	16·76	None	0·91	(Alsberg and Clark)
,,	48·80	7·58	14·75	0·62	1·17	(Present work)
Avian egg-yolk						
Vitellin	51·24	7·16	16·38	0·94	1·04	[Osborne and Campbell, 1900]

(These figures are corrected for ash, which in the preparations described here amounted to 1·42 %.)

Attention was next directed to the dilute salt solution from which the icthulin had been precipitated, and on evaporating down some 10 or 12 litres of this, it was found that another protein was present. Boiling with acetic acid at a p_H just giving a green colour with bromocresol purple produced a flocculent precipitate and trichloroacetic acid also gave a positive result. After half-saturation with ammonium sulphate, however, and filtration of the resulting precipitate, these reagents no longer showed even a trace of protein. A second half-saturation with ammonium sulphate also gave a negative result. The precipitate, however, gave strong xanthoproteic, biuret, and glyoxylic tests, with weak Millon and weak Molisch reactions, and the obvious probability was that here was a protein in selachian yolk corresponding to avian livetin, just as the icthulin corresponded with the vitellin. In order to purify this material further, it was dissolved in water and reprecipitated four times by half-saturation with ammonium sulphate, after which it was boiled with alcohol under a reflux condenser in the same way as the icthulin. It was then

placed in a collodion sac, brought to p_H 5·0 and dialysed against distilled water for about a week, the water being frequently changed, at the end of which time no more ammonia or sulphate could be found in the dialysate. The protein was then dried *in vacuo* over sulphuric acid, and its elementary composition determined, as shown in Table II, under the suggested name of "thuichthin."

Table II.

	C %	H %	N %	P %	S %	
Selachian egg-yolk						
Thuichthin	45·60	7·39	12·14	0·039	1·96	(Present work)
Avian egg-yolk						
Livetin	—	—	15·35	0·067	1·80	(Kay and Marshall)

(These figures are corrected for ash, which in the preparations described here amounted to 1·16 %.)

The second protein of selachian yolk, therefore, presents a close analogy with the second protein of avian yolk, being low in phosphorus and high in sulphur. The N : P ratio is, according to Kay and Marshall, 1 g. nitrogen to 3·14 mg. phosphorus in the case of livetin, in thuichthin it is 1 g. nitrogen to 3·08 mg. phosphorus.

Unfortunately the preparations described here were not made in a sufficiently quantitative manner to enable any statement to be advanced about the relative proportions of icthulin and thuichthin in the dogfish egg. The interest of the results lies rather in the remarkable similarity exhibited by the protein distribution in the eggs of such widely different animals as the hen and the dogfish. It may now be definitely stated that in both these cases the protein of the yolk, as far as can be ascertained by the current methods of protein characterisation, divides into two individual substances, one phosphoprotein and one pseudoglobulin. It would be very interesting to examine the eggs of insects, molluscs, echinoderms, etc., from this point of view, for if these two types of protein alone are to be always associated with the raw material of embryonic development, the question arises, why two should be found there, and not one or half-a-dozen.

SUMMARY.

A study of the yolk-proteins of the dogfish, *Acanthias vulgaris*, allows the following conclusions.

1. The main protein present is a phosphoprotein, containing 0·62 % P, and resembling in its general characters the vitellins.

2. A second protein, to which the name "thuichthin" is given, accompanies the icthulin in this egg. It has the properties of a pseudoglobulin and is analogous to the livetin of avian egg-yolk.

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CXXXVI. THE DEGRADATION OF WOOD BY SIMULTANEOUS ACTION OF ETHYL ALCOHOL AND HYDROCHLORIC ACID.

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(*Received November 6th, 1929.*)

Introduction.

Considerable interest attaches to the fact that by means of acid hydrolysis the carbohydrates of wood can be hydrolysed to sugars, some of which yield alcohol on fermentation. With a view to improving the yields of these fermentable sugars, numerous expedients have been applied, which for the most part have taken the form of variations in acid concentration, temperature, pressure, and duration of acid treatment, while at the same time attempts have been made to catalyse the hydrolysis. Detailed studies of the possible catalytic effect of a range of substances on the acid hydrolysis of wood have been made by Sherrard and Gauger [1923], but although positive effects were obtained in some instances, in no case were the yields of fermentable sugars increased by more than a small amount.

The nature of the results obtained by the above authors has stimulated interest in the effect on wood of other substances in the presence of acids. Of these ethyl alcohol has invited study, in view of its curious, and as yet unexplained, action on plant tissues in the presence of hydrochloric acid.

Mangin [1890], in his studies of the nature and composition of plant cell walls, used a solution of hydrochloric acid in ethyl alcohol as a macerating agent. He claimed that this solution was effective in dissolving the middle lamella of herbaceous plants, with the consequent separation of adjacent cells.

Holmberg and Runius [1925] have investigated the extract obtained from wood by acid-alcohol solutions, and claim that it contains lignin in solution. Moreover, as a result of their investigations, they advance the view that the lignin and cellulose in wood are chemically combined. Friedrich [1928] has also examined the acid-alcohol extract from wood with a view to finding the best conditions for obtaining the so-called soluble lignin. This author characterises the work of Holmberg and Runius [1925] as unconvincing, and produces evidence to show that chemical combination between the lignin and cellulose in wood has not been proved.

In the light of the above facts it has seemed desirable to investigate more closely the effect of acid-alcohol solutions on wood, for no satisfactory data are available with regard to the composition of the wood residue remaining after treatment, and its relationship to that of the original sound wood. Moreover, there would appear to be some reason for assuming that the term "soluble lignin" has been somewhat loosely applied to a precipitate obtained from the wood extract, whose relationship to the parent wood substance has not been fully established. In the present investigation an attempt has been made to deal with some aspects of the wood-acid-alcohol reaction which have been overlooked in previous work.

Material.

The material used was Sitka spruce heartwood sawdust of 60–80 mesh taken from the sample used in a previous investigation by Hawley and Campbell [1927], its composition (Table I) being determined by the analytical methods recommended by Schorger [1926, p. 505] and Hawley and Wise [1926]. The solubility in alcohol was determined by extracting a weighed sample for 6 hours at the boiling-point.

The results throughout are expressed as percentages by weight of the original oven-dry wood.

Table I. *Composition of original wood.*

Water-soluble	4·1
1 % alkali-soluble	12·0
Alcohol-soluble	2·9
Cellulose	62·5
Lignin	29·3
Total pentosans	9·9
Pentosans not in cellulose	6·2

Experimental.

Effect on air-dry wood of a solution consisting of one volume of concentrated aqueous hydrochloric acid and four volumes of 95 % ethyl alcohol.

In the first instance an acid-alcohol solution was prepared by mixing one volume of aqueous hydrochloric acid of Sp. Gr. 1·18 with four volumes of 95 % ethyl alcohol.

A series of six samples of the air-dry wood were thoroughly mixed with the reagent. One sample was maintained at room temperature for 24 hours and the remainder of the series were maintained at 100° over a time range of from $\frac{1}{2}$ to 6 hours. In each case a deep reddish brown extract was obtained, the colour becoming more intense as the treatment increased in severity. After filtering off the extract the residue was thoroughly washed with 95 % ethyl alcohol and then with water until free from acid. The material was then dried at 105° and analysed (Table II).

Table II. *Analysis of wood residue after treatment with acid-alcohol solution.*

Treating time and temperature	Loss due to treatment	Residue					
		Alkali-soluble	Cellulose	Lignin	Methoxyl	Total pento-sans	Pentosans not in cellulose
24 hours at 20°	6·8	11·2	61·2	25·5	4·7	7·6	4·4
½ hour at 100°	27·0	9·5	48·6	20·1	4·0	3·9	2·1
1 hour at 100°	32·7	8·8	44·5	18·3	3·8	3·0	1·6
1½ hours at 100°	33·7	7·14	46·2	17·8	3·42	2·7	0·3
3 hours at 100°	38·9	5·23	44·0	16·3	3·03	1·8	0·5
6 hours at 100°	43·6	5·8	37·6	14·9	2·7	1·3	0·4

Effect of ethyl chloride on oven-dry wood.

Since ethyl alcohol and hydrochloric acid react on heating to form ethyl chloride and water, the following experiments were carried out to determine firstly the effect of ethyl chloride on wood, and secondly the influence of water on the wood-acid-alcohol reaction.

40 cc. of pure ethyl chloride was prepared by the action of heat on ethyl alcohol and hydrogen chloride in the presence of zinc chloride, and 5 g. of the original wood, dried at 105°, was incorporated with this and maintained for 1 hour at 12·5°, the boiling-point of ethyl chloride. After removal of the ethyl chloride, washing with cold alcohol, and drying at 105° there was no loss in weight.

Effect of a weak acid-alcohol solution on oven-dry wood, in the absence of water.

An acid-alcohol solution was prepared by adding 23·9 cc. of hydrochloric acid of Sp. Gr. 1·18 to 1 litre of absolute ethyl alcohol. The acid concentration with respect to the alcohol was thus approximately 1 %. To this solution a large excess of fused zinc chloride was added. A 25 g. sample of the original wood, dried at 105°, was extracted with the solution in a Soxhlet apparatus for 3 hours, absence of water from the extraction thimble being secured by continuous contact of the boiling solution in the flask with zinc chloride. The extract had the characteristic red colour. The residue was washed with alcohol till free from acid, dried at 105°, and analysed (Table III). The loss in weight sustained by the sample during treatment was determined, first, directly, by comparing the dry weight of the residue with that of the original sample, then, by calculation, by comparing the data with those in Table I. The sum of the losses in cellulose, lignin and pentosans not in the cellulose gave the value for "calculated loss" recorded in Table III.

Table III. *Analysis of wood residue after extraction with 1 % acid-alcohol solution in absence of water.*

Loss due to treatment (determined) ...	3·8
Calculated loss	4·2
Cellulose	59·9
Lignin	29·0
Total pentosans	7·8
Pentosans not in cellulose	4·9

Effect of acid-alcohol solution on oven-dry wood in the presence of the water of reaction only.

An acid-alcohol solution was prepared by passing dry hydrogen chloride into cooled absolute alcohol. From this a solution of 8·36 % strength was prepared, and 25 g. of oven-dry wood were treated at 100°. The residue was washed as before, dried at 105°, and weighed; a loss of 51·5 % was sustained, and the lignin content had fallen to 12·6 % by weight of the original oven-dry wood.

A 29·5 % acid-alcohol solution was prepared by saturating absolute alcohol with dry hydrogen chloride at 15°. A 50 g. sample of the original wood, dried at 105°, was treated with 500 cc. of this solution for 1 week at 15°. A partial analysis of the residue is given in Table IV.

Table IV. *Analysis of wood residue after treatment with 29·5 % anhydrous acid-alcohol for 1 week at 15°.*

Loss due to treatment	58·9
1 % alkali-soluble	2·3
Cellulose	26·9
Lignin	12·1

Effect on wood of mild acid hydrolysis, followed by extraction with 95 % ethyl alcohol.

A sample of the original air-dry wood was hydrolysed for 6 hours with 1·5 % aqueous hydrochloric acid. The residue was washed with cold water till free from acid, and extracted with boiling 95 % alcohol for a further 6 hours. After washing, and drying at 105°, the loss in weight was determined, as well as the cellulose and lignin contents (Table V).

Table V. *Analysis of wood residue after hydrolysis with 1·5 % HCl followed by extraction with 95 % alcohol*

Loss due to treatment	20·9
Cellulose	49·7
Lignin	26·4

A hydrolysis similar to the above with 8 % acid followed by extraction with alcohol resulted in a total loss of 29·9 %, and the cellulose content of the residue was 45·7 % by weight of the original dry wood.

Discussion.

In mild aqueous acid hydrolysis of wood there are three outstanding features. Carbohydrates are the most seriously attacked; lignin is affected only to a slight extent, and thirdly, as a direct result of hydrolysis, the residue becomes more soluble in alkali than the original wood. Comparison of Tables I and II shows that hydrolysis does not run true to type when ethyl alcohol is added to the hydrolysing medium. Carbohydrates are attacked as before, but at the same time lignin is largely removed, and the wood residue is less soluble

in alkali than the original wood. Clearly alcohol alone could not be responsible for the removal of so large a proportion of lignin, for according to Schorger [1926, p. 507] the alcohol extract of coniferous woods contains only tannins, linoxyn, highly oxidised resins, and substances related to lignin. In any case it has been shown above that after extraction with boiling alcohol for 6 hours the wood used in this investigation only lost 2·9 % of its oven-dry weight. There remains the possibility that lignin depletion may here be due to the action of ethyl chloride, but experimental evidence has been given to show that wood suffers no loss in weight when treated with this substance alone at atmospheric pressure. As ethyl chloride is volatile above 12·5° and is immiscible with water it can be concluded that it plays no part in the reaction.

Ethyl alcohol alone could not affect the carbohydrates of wood to any marked extent; dilute hydrochloric acid alone could not deplete lignin to the extent shown in Table II. Hence it follows that when wood is treated with an acid-alcohol solution at least two main reactions must take place simultaneously. First, there is hydrolysis of the carbohydrates influenced by the presence of alcohol, and secondly, there is action on lignin by alcohol, solution or combination, influenced by the presence of hydrogen chloride. Both reactions are influenced by the presence or absence of water.

Influence of alcohol on the acid hydrolysis of wood.

The results in Table II show that the hydrolysis has followed a different course from that followed in mild aqueous acid hydrolysis. Thus, on comparing the composition of the residue after treatment in this way for 6 hours at 100° with the composition of the residue from a sample of the same original wood which has been treated with 8 % aqueous hydrochloric acid for the same time at 100° [Hawley and Campbell, 1927], it is evident that the presence of alcohol in the hydrolysing medium has had the effect of increasing the percentage depletion of cellulose and pentosans, including the pentosans in the cellulose. The effect on the lignin of the wood will be discussed later. The most striking result of the addition of alcohol to the hydrolysing medium, however, is that the alkali-solubility of the residue decreases with increasing severity of treatment. It must therefore be concluded that the low alkali-solubility of the residue is partly due to the fact that the carbohydrate hydrolysis products have been removed with the acid-alcohol extract. It has been shown in previous work that after mild aqueous acid hydrolysis some of the products of hydrolysis are left in the residue. Evidence of this deviation from the normal course of acid hydrolysis caused by the addition of alcohol is supplied by the data in Tables I and II.

In Table I the sum of the main constituents, namely cellulose, lignin and pentosans not in cellulose, amounts to 98·0 % of the oven-dry weight of the original wood. In Table II the sum of the same constituents including the loss due to treatment amounts on the average to 97·86 % of the oven-dry weight of the original wood, the extreme variations in the series of six analyses being

96·5 % and 99·7 %. The close agreement of these totals proves conclusively that the residues contain nothing which cannot be estimated as cellulose, lignin or pentosans. Part of the decrease in the alkali-solubility of the residues is thus accounted for at once, while the removal of lignin from the wood accounts for still another portion. The question now arises as to whether the carbohydrate hydrolysis products of the action of acid-alcohol on wood are removed in the extract in a state of solution in, or combination with, alcohol.

If solution of the hydrolysis products alone takes place, then such solution should conceivably succeed hydrolysis, and be independent of acid. Experimental evidence, however, does not bear this out. Hawley and Campbell [1927] have shown that hydrolysis of the original wood with 1·5 % aqueous hydrochloric acid produces a loss of 17·6 % of its oven-dry weight. The results in Table V show that if the residue from a 1·5 % acid hydrolysis be extracted with alcohol for 6 hours the total loss due to the successive treatments amounts to 20·9 % of the oven-dry weight of the wood. Alcohol must therefore have removed 3·3 %, and thus the hydrolysis can only have increased the alcohol-solubility by 0·4 %. In the same way it can be shown that 8 % aqueous acid hydrolysis can only increase alcohol solubility by 2·1 % at the most. It thus becomes evident that during the acid-alcohol treatment of wood, the hydrolysis products must for the most part react with the alcohol in the presence of acid to form soluble compounds, which may possibly be of a glucosidic nature.

The action of alcohol on lignin in the presence of hydrochloric acid.

The comparatively slight effect of mild aqueous acid hydrolysis on the lignin of wood has been demonstrated in previous work. The results in Table II show that the addition of ethyl alcohol to the hydrolysing medium causes marked depletion of this constituent. In view of the fact that in wood analysis the lignin determination is always preceded by extraction of the samples with alcohol-benzene, it has not been shown previously whether the small lignin depletion noted after mild acid hydrolysis is due to solubility of lignin in alcohol as a result of hydrolysis. It has been shown above, however, that 1·5 % aqueous acid hydrolysis only increases alcohol solubility by 0·4 %, whereas comparison of Tables I and V shows that the extent of lignin depletion amounts to 2·9 % by weight of the oven-dry wood. In the same way it can be shown by reference to the data of Hawley and Campbell [1927] that after hydrolysis with 8 % hydrochloric acid the percentage of lignin depletion is in excess of the percentage increase in alcohol-solubility. It must therefore be concluded that the small lignin depletion determined after mild acid hydrolysis takes place during hydrolysis and not during the alcohol-benzene extraction which precedes the lignin determination in the analysis of the residue.

From the foregoing evidence it follows that, when wood is treated with acid and alcohol simultaneously, hydrolysis of the carbohydrates and of a portion of the lignin is independent of the action of alcohol in the presence of acid on the main body of the lignin. This contention is further strengthened

by the experimental data in Table II, which show that when wood is allowed to react with acid-alcohol solution at 20° for 24 hours the degree of carbohydrate hydrolysis is small in comparison with the extent of lignin depletion. It is therefore evident that in the presence of hydrochloric acid, alcohol reacts with a portion of the lignin in wood to form a soluble product, the rate of formation of which does not synchronise with the rate of hydrolysis of the carbohydrates. Holmberg and Runius [1925] have suggested that the formation of such a product is evidence of the presence of an acetal linkage between the lignin and cellulose in wood, and, further, that lignin is the carbonyl component of the acetal. In the wood-acid-alcohol reaction it is assumed that the ethyl group of the alcohol replaces cellulose in this acetal to give a soluble product. The results obtained in this investigation, however, are not consistent with such a suggestion. On the contrary, they rather favour the view of Friedrich [1928] that chemical combination between the lignin and cellulose in wood has not been proved.

The rôle of water in the wood-acid-alcohol reaction.

Holmberg and Runius [1925] have shown that when wood is treated with acid-alcohol containing water, the water has the effect of diminishing the rate of reaction. On the other hand, it has been shown above that the presence of some water is essential to the reaction, for the results in Table III show that, when precautions are taken to exclude water, attack on the wood is similar in effect to mild aqueous acid hydrolysis in that carbohydrates are depleted most and lignin is only affected to a slight extent. The clo , agreement in acid-alcoholic hydrolysis between the determined percentage loss and the calculated percentage loss is due, as shown above, to interaction between the carbohydrate hydrolysis products and alcohol.

It has also been shown above that the maximum effect of acid-alcohol on wood is secured when oven-dry wood is treated with a concentrated solution of dry hydrogen chloride in absolute alcohol. In this way the presence is obtained in the reaction mixture of the minimum amount of water. Friedrich [1928] has shown that the conditions most favourable to the attack on lignin are a long reaction time at a low temperature, coupled with a low water content of a concentrated acid-alcohol solution.

The acid-alcohol extract of wood.

The foregoing experimental evidence shows clearly that the extract obtained from wood by treatment with solutions of hydrochloric acid in ethyl alcohol under various conditions is in every case derived from portions of all the main constituents of wood substance. The results illustrate the fact that acid-alcohol cannot be regarded as a specific solvent for lignin. Holmberg and Runius [1925] claim that neutralisation of the extract with sodium hydroxide effects the precipitation of a substance to which they have applied the term "alcoholysis lignin," but in the light of the results of this investigation it

would appear that the term lignin cannot safely be applied to such a substance until its relationship to the parent wood substance has been fully established, in view of the possibility that it may reasonably be expected to contain material derived from wood constituents other than the lignin proper.

SUMMARY.

1. The simultaneous effect of ethyl alcohol and hydrochloric acid on the wood of Sitka spruce has been examined in detail.

2. Reasons are given for concluding that at least two main reactions are involved, namely, hydrolysis of carbohydrates, influenced by the presence of alcohol, and action of alcohol on lignin in the presence of hydrochloric acid.

3. Absence of water from the system favours depletion of carbohydrates, the maximum effect on the lignin in wood being secured by treating the oven-dry material with a concentrated solution of hydrogen chloride in absolute alcohol, the necessary water being formed by interaction of the acid and alcohol.

4. Experimental evidence is given which favours the view that the association of lignin and cellulose in wood is of a physical rather than a chemical nature.

5. It is shown that the extract obtained from wood after treatment with acid-alcohol solutions under various conditions is in each case derived from portions of all the main constituents, so that the precipitate obtained on neutralisation which has been termed "alcoholysis lignin" may reasonably be expected to contain material derived from wood constituents other than lignin.

The author wishes to express his indebtedness to Dr L. F. Hawley for facilities and advice afforded at the U.S. Forest Products Laboratory, to Prof. F. Soddy for facilities afforded in the Old Chemistry Department, Oxford, to Sir James Irvine for criticising the manuscript, and to R. S. Pearson, Esq., for permission to publish these results.

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CXXXVII. THE CHEMISTRY OF OESTRIN.

II. METHODS OF PURIFICATION.

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(*Received October 7th, 1929.*)

Introduction.

Until quite recently the lack of an accurate method of assaying oestrus-producing extracts has resulted in most of the work on the preparation and purification of the oestrus-producing hormone being qualitative rather than quantitative.

Workers have been of necessity content with purely empirical methods of preparation and purification, which, although yielding extracts that have been highly potent, have also provided evidence on the chemical nature of the hormone which has undoubtedly been misleading.

Recent publications on methods of assaying the hormone by Coward and Burn [1927], Allan, Dickens, Dodds and Howitt [1928], Kahnt and Doisy [1928] and Marrian and Parkes [1929] have now made quantitative work on the hormone a possibility.

In this communication an attempt has been made to repeat and extend much of the previous work on the stability and purification of oestrin with a view to improving the methods of preparing highly concentrated extracts and throwing more light on the chemical nature of the hormone.

Method of assay.

The method previously described by Marrian and Parkes [1929] has been used throughout this work. Since the publication of this paper numerous tests have been carried out and, provided that the described precautions are observed, the error has been found to be little, if any, more than $\pm$ 10 %. No necessity for "priming" the animals as suggested by Kahnt and Doisy [1928] has been found. Provided the animals are injected once a fortnight with not more than 1·5 mouse unit (m.u.) the response is satisfactorily regular.

Standard preparation of oestrin. In a previous communication [Marrian, 1929] the preparation from the urine of pregnancy and initial standardisation of several batches of oestrin were described. These preparations, details of which are shown in Table I, have been used throughout this work.

Table I.

Batch	Type of preparation	Weight of 1 m.u. (mg.)	M.u. per cc. solution
S.B. 1	Aqueous solution	0·0016	37
S.B. 2	Aqueous solution	—	41
E.P.U.	Alcohol-chloroform solution of unsaponifiable matter	0·0183	51
P.U. 4	Unsaponifiable matter	0·00357	—

Samples of the first three batches were used when it was desired only to study the effect of an experiment on the oestrin content of the solution, while batch P.U. 4 was used to investigate the distribution of the total solid matter in relation to the distribution of the hormone in purification processes.

Experimental.

Fractionation with dilute alcohol.

According to several workers, oestrin is soluble to a considerable extent in water-alcohol mixtures [cf. Ralls, Jordan and Doisy, 1926].

If it is assumed that the hormone is a true water-soluble substance, there would seem to be no serious objection to eliminating all the definitely water-insoluble substances by fractionation with alcohol-water mixtures of decreasing strength until only true water-soluble material remains.

The following series of experiments was carried out in order to see how far the dilution of the alcohol could be carried without loss of potency.

(*a*) 70 % *alcohol.* Batch P.U. 4, consisting of 1·106 g. of the unsaponifiable ether-soluble fraction from 164 litres of urine, was heated to boiling with 30 cc. of 70 % alcohol for 5 minutes. A considerable amount remained undissolved. The mixture was allowed to stand at 0° for several hours and then filtered. The precipitate on the filter was washed through into the original flask containing the insoluble material with boiling absolute alcohol. After evaporation of the absolute alcoholic solution, the insoluble material was treated twice in the same manner with 15 cc. of 70 % alcohol.

After these three extractions, there finally remained 0·563 g. of insoluble material of which 44 % was cholesterol.

The combined 70 % alcoholic filtrates were evaporated to dryness *in vacuo* and yielded 0·507 g. of a reddish brown semi-solid gum.

Assay. The gum was dissolved in 250 cc. of chloroform. From 1 cc. of this solution the chloroform was removed and the residue made up to 10 cc. with alcohol. After preliminary tests 0·22 cc. of this solution was diluted to 10 cc. with water and injected into a group of mice in four doses of 0·1 cc. each. 58 % of the mice gave a full oestrous response. This corresponds to a dose of 1·07 m.u. per mouse or a total number of 303,900 m.u., the weight of 1 m.u. being 0·00167 mg. Since the original material was assayed to contain 309,500 m.u., it is evident that no detectable loss of activity had occurred, while considerable purification had been effected.

(*b*) 50 % *alcohol.* The 70 % alcohol-soluble fraction was treated in an exactly similar manner with 50 % alcohol. In this way 0·335 g. of soluble material and 0·167 g. of insoluble material were obtained. Since only 9 % of the latter consisted of cholesterol, it is probable that the fraction soluble in 50 % alcohol was practically cholesterol-free. This fraction was indistinguishable in appearance from that soluble in 70 % alcohol.

Assay. Working on the assumption that all the activity was in the soluble fraction, the testing was carried out using the same dilutions as in (*a*). 61 % of a group of mice showed a full oestrous response, corresponding to a total of 315,300 m.u., and a mouse unit weighing 0·00106 mg. Again it was evident that there had been no loss of activity.

(*c*) 30 % *alcohol.* By treatment in the same way with 30 % alcohol, 0·133 g. of soluble and 0·206 g. of insoluble material were obtained.

Assay. Preliminary tests showed that the soluble fraction only contained part of the total activity. Both fractions were therefore made up to 250 cc. in alcohol. 1 cc. of the soluble fraction was diluted to 10 cc. with water and injected in four doses of 0·1 cc. each. 20 % of a group of mice gave an oestrous response, corresponding to 0·83 m.u. per mouse, or a total of 86,450 m.u. The weight of one mouse unit of this fraction was therefore 0·00154 mg.

0·3 cc. of the solution of the insoluble fraction was diluted to 10 cc. with water and injected in four doses of 0·1 cc. each. Oestrus was shown by 60 % of the group of mice. This corresponds to 1·09 m.u. per mouse and a total of 227,000 m.u. The weight of one mouse unit of this fraction was 0·000865 mg.

The total mouse units in each fraction add to 313,450 which is in satisfactory agreement with the original amount of 315,300 m.u. This result could be explained in one of two ways, either the hormone has only a low solubility in 30 % alcohol or it has a high solubility but is readily adsorbed by filter-paper from 30 % alcohol. In the latter case, which seems possible in view of the statement of Laqueur, Hart and de Jongh [1927] that oestrin is readily adsorbed by filter-paper from aqueous solutions, the adsorbed hormone would be washed back with the insoluble fraction on washing the filter with boiling alcohol. In either case, it is evident that fractionation with 30 % alcohol does not provide an efficient means of purification.

Resistance to alkali treatment and extraction from aqueous media by ether.

That oestrin may resist boiling for long periods with strong aqueous alkalis or sodium ethoxide solutions seems to be clear from the work of Ralls, Jordan and Doisy [1926] and Laqueur, Hart and de Jongh [1926], although the former experienced losses which they consider may possibly have been due to causes other than hydrolysis. The following experiments entirely confirm these findings.

(*a*) *Aqueous potassium hydroxide.* 1 cc. of batch S.B. 1 was placed in each of two 10 cc. volumetric flasks. 5 cc. of 4·8 % aqueous KOH were added, and the flasks were heated for different periods in a boiling water-bath. To avoid

possible losses by oxidation, a stream of nitrogen was blown over the surface of the liquid. At the end of the experiment, after cooling and exactly neutralising with the predetermined quantity of acetic acid, the contents were made up to 10 cc. These solutions were then injected into groups of mice in four doses of 0·1 cc. each. The results are shown in Table II.

Table II. *Resistance of oestrin to hot aqueous KOH.*

M.u. in each flask before experiment	Time heated (mins.)	Mice on oestrus (%)	M.u. in each flask after experiment
37	40	75	35
37	180	82	39

(*b*) *Sodium ethoxide.* In a second series of experiments, 4·5 cc. of batch E.P.U. were placed in each of two flasks. The solvent was removed by evacuation of the flask at 100° and 5 cc. of 0·2 *N* sodium ethoxide solution were added to each flask. The flasks were then heated on a hot plate to a temperature of 100° under reflux condensers for different times, a stream of nitrogen being played on the surface of the liquid. After neutralisation with the exact amount of acetic acid, the volumes were made up to 60 cc. with water and four doses of 0·1 cc. injected into two groups of mice. The results are shown in Table III.

Table III. *Resistance of oestrin to hot Na ethoxide.*

M.u. in each flask before experiment	Time heated (mins.)	Mice on oestrus (%)	M.u. in each flask after experiment
216	40	85	246
216	120	75	204

These results show quite definitely that oestrin is quantitatively stable to treatment with aqueous alkalis and sodium ethoxide at 100° for at least 2 hours. This does not necessarily mean, however, that crude preparations of the hormone can be satisfactorily purified by ether extraction of the saponified material. On the contrary a considerable amount of evidence is accumulating to show that such a procedure may result in the loss of a considerable fraction of the total activity.

Thus Allan, Dickens, Dodds and Howitt [1928] show that the washing of an ethereal solution of the hormone by aqueous alkali results in a partition of the activity between the two layers. They state that this is due to adsorption by alkali soaps in the aqueous layer. Glimm and Wadehn [1929] claim that boiling with alkali converts the hormone into a form insoluble in ether. Funk [1929] states that oestrin is alcoholic or phenolic in nature and on treatment with alkalis forms salts. He explains the appearance of the hormone in the unsaponifiable matter on the grounds that the alkali salts are soluble to some extent in ether.

Accordingly it was decided to study quantitatively the extraction of oestrin from aqueous media at neutral, acid and alkaline reactions. It is extremely

important to use only freshly redistilled ether of the highest degree of purity for such extractions. Numerous results have been frequently obtained confirming the statements of Thayer, Jordan and Doisy [1928] that the hormone is rapidly destroyed by ether peroxides.

(*c*) *Extraction of neutral aqueous solution by ether.* 50 cc. of batch S.B. 2 containing 2050 m.u. were shaken in a funnel with about 80 cc. of ether in four successive quantities of 20 cc. The combined ethereal extracts were washed once with about 20 cc. of water and evaporated to dryness. The residue was dissolved in about 3 cc. of alcohol and then made up to 50 cc. with water. 0·66 cc. of this solution was diluted to 10 cc. and after injection into a group of mice in four doses of 0·1 cc., produced a 60 % oestrous response. This corresponds to a total of 2065 m.u. Thus the extraction of the hormone from neutral solution by ether was complete.

(*d*) *Extraction of acid aqueous solution by ether.* 5 cc. of E.P.U. solution containing 255 m.u. were evaporated to dryness and then dissolved in about 1 cc. of alcohol. 80 cc. of 0·5 *N* HCl were added and the mixture was shaken out four times with 30 cc. of ether. The combined ethereal extracts, after washing once with 20 cc. of water, were evaporated to dryness, dissolved in 5 cc. of alcohol and made up to 100 cc. This solution was injected into a group of mice in four doses of 0·1 cc. and 35 % of the group showed an oestrous response. This corresponds to a total of 230 m.u., and, within the limits of error of testing, may be considered to represent quantitative recovery of the hormone.

(*e*) *Extraction of alkaline aqueous solution by ether.* 5 cc. of E.P.U. were evaporated to dryness and dissolved in a small volume of alcohol. 80 cc. of 4·8 % aqueous KOH were added and the mixture was then extracted four times with 30 cc. of ether. The combined ethereal extracts were washed once with 20 cc. of water and evaporated to dryness. The residue was dissolved in 3 cc. of alcohol and made up to 100 cc. with water. This was injected in four doses of 0·2 cc. each. The group of mice showed a 20 % positive response, corresponding to a total of 103 m.u.

Since it was expected that ether extraction from alkaline solution might prove to be incomplete, the alkaline solution, together with the washing of the ether extract, was acidified with 6 cc. of 12 *N* HCl and re-extracted with ether as before. The product was made up to 100 cc. as in the previous experiments and injected in four doses of 0·15 cc. each. This resulted in a 30 % oestrous response or a recovery of a further 148 m.u. The total recovery of 251 m.u. is quantitative.

The failure of oestrin to be extracted with ease from aqueous potassium hydroxide solutions by ether would seem to be due to either (*a*) the formation of a potassium salt which is slightly soluble in ether as suggested by Funk [1929], (*b*) the formation of a potassium salt which dissociates to some extent, thus allowing the free dissociated oestrin to dissolve in the ether layer, or (*c*) adsorption by soaps in the aqueous layer, as suggested by Allan, Dickens,

Dodds and Howitt [1928]. The last possibility seems somewhat improbable as the batch E.P.U. was prepared by a saponification process [Marrian, 1929] and should be reasonably free from soaps or easily saponifiable substances. It will be shown in a later experiment that this loss is almost certainly not due to adsorption by soaps.

Since the evidence indicated that oestrin might be acidic in nature as Funk suggests, it was thought desirable to ascertain what effect in the extraction by ether a preliminary saturation of the alkaline solution by carbon dioxide would have. 5 cc. of E.P.U. were treated in a manner identical to that in the last experiment with the exception that carbon dioxide was bubbled through the alkaline solution for 1·25 hours before ether extraction. The residue from the ether extraction was made up to 100 cc. as before and injected in four doses of 0·2 cc. each. An oestrous response of 89 % corresponding to a recovery of 229 m.u. resulted.

This result suggests that oestrin is a weakly acidic substance which can be displaced from combination with alkalis by carbon dioxide, and that the loss on purification by a saponification process might be avoided by such treatment.

Extraction of oestrin from ethereal solution by aqueous alkali.

The low solubility in ether of what may provisionally be called the potassium salt of oestrin and its higher solubility in aqueous alkalis suggested the possibility of completely removing the active principle from an ethereal solution by repeated extraction with aqueous KOH. Since all the preparations used had been prepared by the converse process, *i.e.* extraction from aqueous alkaline solution by ether [Marrian, 1929], it was hoped that considerable purification might be effected in this manner.

In a preliminary experiment, 5 cc. of E.P.U. solution were evaporated to dryness and dissolved in 80 cc. of ether. This solution was shaken four times in succession with 30 cc. of 4·8 % KOH. The combined potash extracts were approximately neutralised with HCl and the ether was removed by evaporation under reduced pressure. The solution, after exact neutralisation to litmus with acetic acid, was made up to 200 cc. and injected in four doses of 0·25 cc. 63 % of the mice gave an oestrous response which corresponds to a recovery of 226 m.u. This experiment clearly shows that from such an ethereal solution the hormone can be completely extracted by aqueous potassium hydroxide.

It was still possible, however, that this might be due to adsorption of the active principle by traces of potassium soaps; accordingly it was decided to repeat the experiment on batch P.U. 4 which was certainly free from soaps or readily saponifiable substances, since during its preparation it had been saponified twice and the soaps had been removed by frequent washing with water [Marrian, 1929]. This experiment would also serve to study the distribution of total solids between the two phases, so that the efficiency of the process as a method of purification could be investigated.

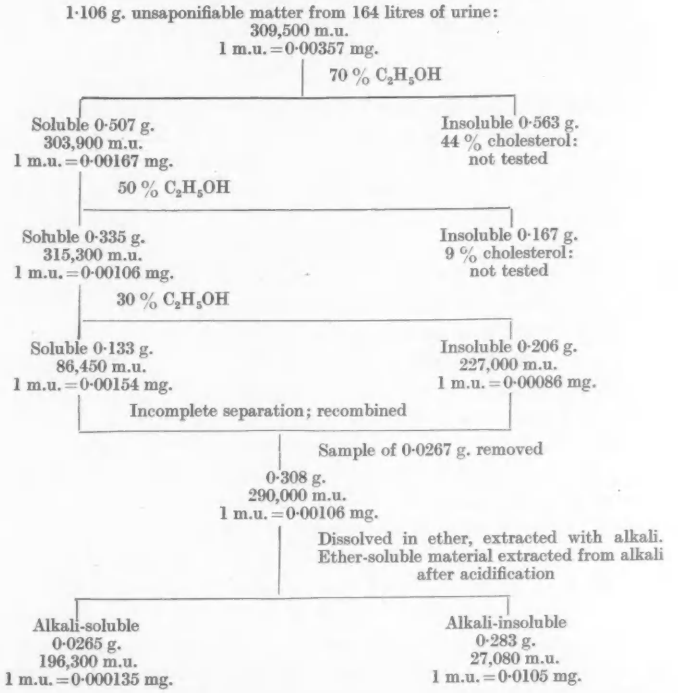
The two fractions of P.U. 4 (soluble and insoluble in 30 % alcohol) were

recombined and made up to a known volume. An aliquot portion was removed for physiological tests, leaving on evaporation 290,000 m.u. and 0·308 g. of solid material. This was dissolved in 500 cc. of ether and extracted in a funnel with seven successive lots of 100 cc. of 4·8 % KOH. The combined alkali extracts, after acidification with 80 cc. of 12 *N* HCl, were extracted twelve successive times with 100 cc. of ether. The combined ethereal extracts, after washing twice with water, were evaporated to dryness and yielded 0·0265 g. of a dark reddish semi-solid that appeared to contain a certain amount of crystalline material.

This was made up to 250 cc. with chloroform. 0·15 cc. of this solution was evaporated to dryness and, after dissolving the residue in 5 cc. of alcohol, this solution was made up to 50 cc. with water. Two groups of mice were injected with the solution in four doses of 0·1 cc. and four doses of 0·15 cc. These two groups showed oestrous responses of 30 % and 80 % respectively, corresponding to a total of 205,100 and 187,500 m.u. From the average of these figures the weight of one mouse unit is 0·000135 mg.

The stages in the purification of batch P.U. 4 are presented in Table IV.

Table IV. *Purification of oestrin: batch P.U. 4.*



The original ether solution, after extraction by the alkali, was washed once with water and evaporated to dryness. 0·2833 g. of a dark red-brown gum was obtained, differing from the other fraction in not appearing to contain any crystalline material. This gum was dissolved in chloroform and made up to a volume of 250 cc. 1·2 cc. of this solution were evaporated to dryness and, after dissolving the residue in 5 cc. of alcohol, this was diluted to 50 cc. with water. A group of mice injected with four doses of 0·1 cc. gave a 55 % positive response, corresponding to a total of 27,080 m.u. in this fraction.

The total recovery of 223,380 m.u. represents a loss of just over 20 %. This is probably well outside the maximum error in the method of assay and must be considered to be experimental loss. Although the separation was not quite so complete as was at first hoped for, the method clearly provides a most efficient means of removal of inactive material from the extracts.

Solubilities of main oestrin fraction.

Attempts were made to obtain material which was definitely crystalline from the oestrin concentrate of 0·0265 g. by recrystallisation. So far, however, no suitable solvent has been found.

The material was extremely soluble in methyl, ethyl and propyl alcohols, chloroform, acetone and ethyl acetate. In ether, benzene, hexane, 30 % alcohol and water the solubility appeared to be extremely small. Laqueur, Hart and de Jongh [1927], working with concentrates obtained from placenta, state that the purest preparations of the hormone (0·0008 mg./m.u.) which they were able to obtain were no longer soluble in the common volatile fat solvents but only in water. In view of these somewhat conflicting observations it must be concluded that present data on the solubilities of the concentrates give little reliable evidence on the actual solubilities of the hormone itself. The observed solubilities must be considered as properties merely of the substances with which the hormone is associated, the nature of which must vary with the method of preparation and the source.

Doisy, Veler and Thayer [1929] found that their method of preparing aqueous solutions of the hormone from extracts of liquor folliculi resulted in failure with urine concentrates. This method, consisting of solution of the concentrate in alcohol, dilution with water and subsequent removal of the alcohol by evaporation under reduced pressure, is essentially the same as that adopted by Allan, Dickens, Dodds and Howitt [1928].

An attempt to prepare an aqueous solution from the main oestrin fraction of 0·0265 g. by this method was only partially successful. After boiling off the alcohol, a large amount of solid material separated. On assaying the oestrin content of the clear supernatant liquid, it was found that about two-thirds of the total activity must have been present in the separated solid. Unfortunately Doisy, Veler and Thayer do not state the exact nature of the

difficulties encountered, but since their product was approximately of the same potency[1] as the one here described, it may be assumed that they were similar.

At present no satisfactory quantitative method of obtaining the activity from urine extracts in a "water-soluble" form has been devised.

Summary.

1. Using the method of assay of Marrian and Parkes [1929] a study of the efficiency of different methods of purifying oestrus-producing extracts from urine has been made on a quantitative basis.

2. By fractionation with 70 % and 50 % alcohol, the activity may be obtained quantitatively in the soluble fraction in each case, with considerable loss of the total solid matter.

3. The high degree of stability of the hormone to boiling aqueous alkalis and sodium ethoxide, found by numerous other workers, has been confirmed.

4. Oestrin can be extracted quantitatively from neutral or acid aqueous solutions by ether with ease, but it is much less readily extracted from alkaline aqueous solution.

5. After passing carbon dioxide into an aqueous alkaline solution of oestrin, the hormone may be quantitatively extracted by ether with ease. This suggests that oestrin is a weakly acidic substance.

6. Oestrin may be extracted from an ethereal solution by aqueous potassium hydroxide. This process removes almost all the activity from the ethereal solution and only a small proportion of the solid matter.

7. By these means a concentrate with an activity of 1 m.u. = 0·000135 mg. has been prepared. The solubilities of this preparation are described.

The author wishes to express his thanks to Prof. J. C. Drummond for continual interest and advice in this work, and to Dr A. S. Parkes for his interest and generous supply of ovariectomised mice.

The expenses of this work were defrayed by a grant from the Medical Research Council.

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[1] They report the preparation from urine of an extract, the rat unit of which is less than 0·001 mg. Since the rat unit is probably from seven to ten times greater than the mouse unit, this preparation must be of about the same degree of potency as the one described in this paper.

CXXXVIII. THE THIOL-DISULPHIDE SYSTEM.

I. COMPLEXES OF THIOL-ACIDS WITH IRON.

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(*Received November 5th, 1929.*)

The addition of iron salts to alkaline solutions of a variety of thiol acids leads to the immediate production of intense red or violet colours. These fade more or less rapidly but may repeatedly be regenerated by bringing the faded solutions into contact with oxygen. This characteristic of thiol acids has long been familiar and has generally been held to indicate the existence of complexes of RSH[1] with both ferric and ferrous ions. The colour has been attributed to an unstable ferric complex which suffers spontaneous autoreduction at the expense of RSH to a colourless, freely autoxidisable ferrous complex. It is assumed, in other words, that these complexes constitute an oxidation-reduction system reversible with respect to the state of oxidation of the iron, the oxidation of RSH being merely an accessory and irreversible change.

The significance of such a hypothesis to the chemical and the biological activity of the RSH—RSSR system is obvious. The high rates at which oxygen oxidises the ferrous complex and RSH reduces the ferric complex provide a simple basis for the definition of the important catalytic influence of traces of iron on the oxidation of RSH and suggest that it may be these which control the interesting relations of glutathione[2] to tissue respiration. This argument

[1] The symbols RSH and RSSR are employed as general descriptive terms for the thiol and disulphide components, respectively, in the systems discussed.

[2] The question of the chemical constitution of glutathione has been re-opened since this paper was written. It now appears that those preparations from natural sources, of a material to which we have been accustomed to give the name "glutathione" and with which the chemical and biochemical behaviour of "glutathione" have been established, were chemically impure. It appears that the chief thiol constituent of this material is a tripeptide [Hopkins, 1929], some of whose biochemical activities differ from those of the impure product [Dixon and Meldrum, 1929]. Such a situation, of course, demands a re-investigation of the biological function of this substance. In the meantime, however, the facts of "glutathione respiration" and the dominant part which the thiol group plays in these phenomena remain unchallenged. In the present paper the name "glutathione" is retained, without structural implications, for the description of those phenomena involving the thiol group which have been disclosed with the aid of impure glutathione.

was first developed by Warburg, whose view seems to be that the essential biological rôle of the thiol group in such compounds as glutathione is the provision of a suitable vehicle for the exhibition of the catalytic activity of iron. The matter is of some importance since the quantitative establishment of the above contention would undoubtedly lead to a reconciliation of many conflicting views of the mechanism of biological oxidation-reduction.

In spite of these implications, however, no quantitative attempt appears to have been made to arrive at the chemical composition of these hypothetical complexes or at the conditions of their formation and reaction. In the present paper such an attempt is described. The data assembled will, in a later communication, be extended towards a quantitative definition of the main kinetic relations of the RSH—RSSR system and of the catalytic influence of iron upon these. Since the completion of the present work there have been published two reports by Michaelis and his colleagues [Michaelis and Barron, 1929, 2; Michaelis and Yamaguchi, 1929] of attempts to arrive at the nature of these iron-thiol complexes by the study of the more stable cobalt-thiol complexes. These interesting studies are discussed in the text.

Qualitative observations.

Discussion will be facilitated by the preliminary description of orienting experiments which serve to define the general relations of iron to RSH—RSSR. These observations actually relate specifically to thioglycollic acid. They may, however, be extended to such substances as thiolactic acid, cysteine and glutathione with only minor modifications affecting equilibrium constants, rates of reaction and relations to p_H. It must be emphasised, at the outset, that the critical requirement of all this work is the rigorous exclusion of oxygen from all reagents and apparatus. This was achieved by thorough deaeration with purified nitrogen.

1. When, under these conditions, ferric ions are added to a solution of RSH buffered within the p_H range 6–11, a deep red colour instantly forms. This proceeds to fade at a rate which increases rapidly with $[H^+]$ between these limits of p_H. At p_H 7 the change is almost instantaneous. The coloured solution, moreover, is not only subject to spontaneous fading, but may be instantly decolorised by such soluble reducing agents as titanous chloride and sodium hydrosulphite. In all cases the colour may be instantly restored by admission of oxygen or by soluble oxidising agents, *e.g.* iodine or methylene blue. The cycle of fading and regeneration may be repeated indefinitely until such time as iron begins to separate from solution. Examination of the solution at this point shows that almost the whole of the RSH has been converted into RSSR.

2. The tendency of ferric ions to form the red colour diminishes rapidly as the p_H is raised above 10. Instead, there is an increasing tendency for the immediate precipitation of the added iron by the anions present.

3. When ferric ions are added to a solution of RSH buffered at a p_H more acid than 4, a transient blue flush replaces the red flush observed when the p_H is raised to about 6. The one colour merges gradually into the other as the reaction is modified between these limits, the colour change appearing to be centred very approximately at p_H 5. The colourless solutions which succeed the blue colour in acid solution yield no colour with oxygen or other oxidising agents. When the acid mixture is made alkaline no colour appears if sufficient care has been taken to prevent the intrusion of traces of oxygen. The familiar red colour is, however, produced as soon as an oxidising agent is added.

4. When ferrous ions are added to RSH no colour appears in either acid or alkaline solution. The mixture behaves towards oxidising agents exactly like the solutions resulting from the decoloration of ferric-RSH mixtures.

5. Apart from the effect of p_H, the most important factors controlling the stability of the red colour are the concentrations of ferric iron and of RSH. Owing to the spontaneous fading of the red colour it is not possible to obtain a precise measure of any stoichiometric relations which may govern the influence of these functions. If, however, we choose a p_H, *e.g.* 10, at which fading is relatively slow, it is possible to arrive at some approximate relations of interest. When solutions of ferric iron in RSH at this p_H are progressively diluted there is, at first, no loss of colour greater than that which can be attributed to dilution. When the concentration of RSH has been reduced to about 0·005 M—the figure varies somewhat with p_H, increasing rapidly above 10—there is a fairly sudden diminution in colour intensity and a simultaneous separation of a precipitate containing iron. The same critical point is obtained if to a suspension of ferric iron in buffer solution increasing amounts of RSH are added.

If, now, we choose a concentration of RSH which is sufficient for maximum colour production we may attempt the determination of the solubility of iron in it. A 0·1 M solution of RSH at p_H 10 was titrated with ferric chloride. The added iron remained in solution and the colour increased in intensity up to a point which corresponded very roughly with an iron: RSH ratio of 1 : 5. Further addition of iron merely resulted in the separation of a precipitate.

The behaviour of ferrous-thiol mixtures was rather different. Iron did not separate from such mixtures even at great dilutions of RSH.

6. Addition of cyanide to ferric-RSH mixtures led to a progressive discharge of the red colour. The resulting colourless solutions were not sensitive to oxygen.

7. RSSR does not appear to be capable of dissolving iron in alkaline solution and no colours are formed. RSSR was unable to oxidise the colourless ferrous-RSH solutions to the red colour.

The foregoing observations appear to give ample justification for the conclusion that both ferrous and ferric ions form complexes with RSH. The qualitative evidence presented suggests that the ferric complex dissociates into its components more readily than does the ferrous complex and is not

inconsistent with the probability that the composition of these complexes is determined by the usual co-ordination number of iron—six. The system ferrithiol-ferrothiol is freely reversible and attains rapid equilibrium with other reversible oxidation-reduction systems. Ferrithiol, moreover, suffers rapid autoreduction at the expense of RSH and ferrothiol is rapidly oxidised by oxygen in neutral and alkaline solution. The velocity of the former process is some direct function, while that of the latter may be some indirect function, of $[H^+]$. Cyanide enters into the composition of one or both of the complexes with modification in the oxidation-reduction behaviour of the system. The ferrithiol probably suffers a change of ionic charge in the neighbourhood of p_H 5. Above p_H 11 it is more or less completely dissociated into its constituents. There is no evidence that complex formation introduces any enhanced degree of reversibility into the more or less irreversible process RSH → RSSR.

It should be mentioned that the above conclusions are contrary to those at which Lyons [1927] arrived as a result of observations on the reaction of iron with thioglycollic acid. Lyons expressed the view that the red colour is due to the coloured ion of a colourless ferrous complex. As Michaelis and Barron [1929, 2] have suggested, however, this erroneous conclusion arose through insufficient care being taken by Lyons to ensure that all trace of oxygen was excluded from the reaction mixtures which he regarded as being free from oxygen.

We have been unable to isolate in the solid state any of the complex compounds which may be present. Consequently we must be content to study their behaviour in systems in which the concentration of RSH and of iron are controlled. An attempt, on this principle, to give some quantitative definition of the kinetic and equilibrium relations which have been indicated above will now be made.

The autoreduction of ferrithiol.

A series of velocity curves for this reaction at p_H values from 8 to 10·5 are given in Fig. 1 (p. 1249). Attempts to extend the experimental range of p_H failed owing to the great velocity of the reaction in solutions of greater $[H^+]$, and to the separation of a precipitate of iron beyond the more alkaline extreme. The method consisted in the determination at intervals of the amount of residual red colour (by titration with sodium hydrosulphite) in an oxygen-free reaction mixture originally constituted by adding a known amount of ferric ions to a buffered solution of RSH. Since no precipitates were observed in the reaction mixtures of any of the experiments quoted, we are entitled to assume that the whole of the iron which had been added had entered into complex formation. Indeed, the analysis of the electrometric data which is presented in a later section of this paper indicates definitely that the initial concentration of ferrithiol should have been equal to the amount of iron added. That is to say, we are entitled to assume a common origin to the several reaction curves. In such case it is clear that the reaction follows an unusual

course. The character of the curves is such as to suggest a process moving towards an equilibrium which is short of complete change of the initial reactant. This would imply that the reaction

$$2 \text{ ferrithiol} + \text{RSH} \rightarrow 2 \text{ ferrothiol} + \text{RSSR}$$

was significantly reversible. Control experiments seem, however, to have excluded this explanation. Fig. 1 indicates that around p_H 10 the hypothetical reverse reaction should proceed at a rate comparable with that of the forward process. Nevertheless a ferrithiol solution which had been allowed to become completely decolorised at p_H 8 did not develop any colour during several hours after the p_H had been adjusted to 10 in the absence of oxygen. The addition of a high concentration of RSSR to a ferrothiol solution at the latter p_H did not lead to any development of colour.

Our observations are admittedly subject to some important sources of error. A more suitable choice of relative concentrations of reactants might have been made had the results of electrometric studies been then available. The temperature was not closely controlled. Moreover, in spite of the fact that the molar concentration of the buffer was adjusted to 25 times that of the iron, it may be that minor changes in p_H due to the progress of the reaction were not completely eliminated. Finally, there was a source of error which was not appreciated at the time. Michaelis and Barron [1929, 1] have shown that the oxidation of RSH by oxygen and by methylene blue is catalysed by mercury. It is probable, therefore, that its oxidation by ferrithiol is similarly catalysed. Now, the majority of our reaction mixtures were stored in protected burettes over mercury. We have, indeed, had occasion to suspect that in the mixtures which had been stored for long periods before titration, the layer of solution immediately adjacent to the mercury decolorised more quickly than the bulk of the solution. In spite of these sources of error, however, we are satisfied that the general form of the reaction curves is significant and, therefore, the description of them by means of an equation becomes a matter of convenience even if the latter is nothing more than an empirical correlation. We were, however, interested upon certain theoretical grounds, to test the validity of the relation

$$\frac{dx}{dt} = k\left(\frac{x}{a-x}\right)^2 \qquad \text{.....(1)}$$

which may be integrated to

$$kt = 2a\ln\cdot\frac{a}{x} + \frac{x^2-a^2}{x},$$

a being the initial concentration of ferrithiol and x the concentration remaining after time t. Table I indicates the degree to which a typical velocity curve conformed to this equation. If, for purposes of further analysis, we accept this order of reaction, the relation of k to p_H may be defined. The relation is displayed in Fig. 2. Within the experimental range of p_H this figure suggests that k varies with $[H^+]^2$. Further discussion of this reaction is postponed until a later communication.

Table I. *Rate of autoreduction of ferrithiol.*

0·0018 *M* Fe‴. 0·0098 *M* RSH. 0·05 *M* carbonate buffer. p_H 9·0. Room temperature. 10 cc. samples titrated with 0·0042 *M* hydrosulphite.

$kt = 2a\ln\frac{a}{x} + \frac{x^2 - a^2}{x}$, $a = 1$ and x = fraction of original titre.

t (mins.)	Titre (cc.)	x	k
—	4·35	1·0	—
(1·25)	(1·40)	(0·32)	(0·4)
2·5	1·27	0·292	0·27
5·5	0·99	0·228	0·22
6·5	0·92	0·212	0·22
6·0	1·13	0·260	0·15
8·0	0·99	0·228	0·15
10·0	0·91	0·209	0·15
14·5	0·66	0·152	0·18
17·5	0·60	0·138	0·18
23·5	0·50	0·115	0·18
36·0	0·38	0·087	0·18
54·0	0·20	0·046	0·29

The rate of autoxidation of ferrothiol.

This is the reaction which, with the autoreduction of ferrithiol, must be assumed to control the catalytic efficiency of iron in the autoxidation of RSH. It is desirable, therefore, that there should be information on the rate of this reaction and on its variation with p_H. Unfortunately, it has not proved possible to make direct measurements of this velocity, since it is precisely within that range of p_H at which autoreduction of ferrithiol is most rapid that the autoxidation of ferrothiol proceeds at rates convenient for measurement. Only broad generalisations are possible.

Above a p_H of about 8 the autoxidation of ferrothiol, is, undoubtedly, very rapid. It even exceeds in rate the very rapid reverse process of autoreduction since solutions of RSH and iron maintain a deep colour when in equilibrium with partial pressures of oxygen as low as 10 mm. mercury. Below a p_H of 6, on the other hand, it is not possible to demonstrate any autoxidation of ferrothiol. It might be objected that this is due merely to the overwhelming rate at which the reverse reaction has been shown to proceed at this p_H. If this were the situation, however, it would follow that the autoxidation of RSH in the presence of iron should be almost instantaneous in acid solution. Actually, the catalytic influence of iron on the reaction diminishes rapidly as one passes to the acid side of neutrality. We must conclude, therefore, that the autoxidation of ferrothiol is some fairly high function of $[OH^-]$.

Electrode potentials of the system ferrithiol-ferrothiol.

Notation.

$[S]$ = the total concentration of RSH.

$[S']$ = the total concentration of RSH not in combination with iron.

$[Fe'']$ = the total concentration of iron present in the system in the state of lower (ferrous) valency.

$[Fe''']$ = the total concentration of iron present in the system in the state of higher (ferric) valency.

K'' = the dissociation constant of the ferrous complex.
K''' = the dissociation constant of the ferric complex.
k_a = the hydrion dissociation constant of the thiol group of RSH.
k_b = the hydrion dissociation constant of the tervalent cation of the ferric complex [Bronsted, 1923].
E_h = the observed potential referred to that of the normal hydrogen electrode.
E_0, E_1, E_2, E_3, etc. = working electrode constants.

It is well known that solutions of "pure" thiol compounds will induce negative potentials at electrodes of gold, platinum and mercury. All observers are, however, agreed that the electrode behaviour of RSH is anomalous and capricious. Persistent and often irregular drifts are the rule, potentials are easily displaced by mild polarisation of the electrodes and may recover only slowly. Electrodes of different metals are inclined to record grossly different potentials and even "identical" electrodes will give concordant results only with strict standardisation of technique. At best, one does not attempt to record observations more accurately than to the nearest millivolt. Actual reproducibility of independent experiments is of a still lower order [cf. Michaelis and Flexner, 1928].

With the addition of ferric ions and the appearance of the red colour there is an immediate improvement in electrode response. The discrepancies between grossly discordant electrodes are greatly reduced and, generally, quite eliminated. Electrodes withstand polarisation well and recover rapidly. The potentiometer may be balanced confidently to the nearest 0·1 mv. Drifts may still persist but they are similarly recorded by different electrodes and are of a character to suggest changes in the system rather than idiosyncrasies of the electrodes. There can be little doubt that complex formation has induced a degree of electromotive activity previously absent.

In reaction mixtures in which we had reason to believe that the whole of the iron present was dissolved in the form of complexes, it soon became apparent that we were dealing with a system whose potential was determined by the ratio of the concentrations of ferrous and ferric iron. This was established in the usual way by varying the ratio while the p_H and the concentration of RSH were held relatively constant. Two methods were employed. In the first, ferrithiol was titrated with sodium hydrosulphite and in the second, ferric ions were added progressively to a solution of ferrothiol. Now, the application of these methods is complicated by the spontaneous autoreduction of the oxidant. When this is proceeding at a significant rate the experimentally established equilibrium mixture will be subject to continuous change and the determination of equilibrium potentials will be prejudiced by persistent negative drifts. Nor is the order of the reaction such that these drifts can be usefully extrapolated to zero time. The order of the reaction, on the other hand, is of such a character that it diminishes very rapidly with its own progress, and, as Fig. 1 indicates, the reaction above p_H 10 is virtually arrested before 50 %

of the ferrothiol has been reduced. By carrying out titrations at this p_H, therefore, it was possible to reproduce considerable sections of complete titration curves without the states of equilibrium being affected by the secondary reaction. Unfortunately, this situation only holds for thioglycollic acid. The autoreduction of ferricysteine and of ferriglutathione is so

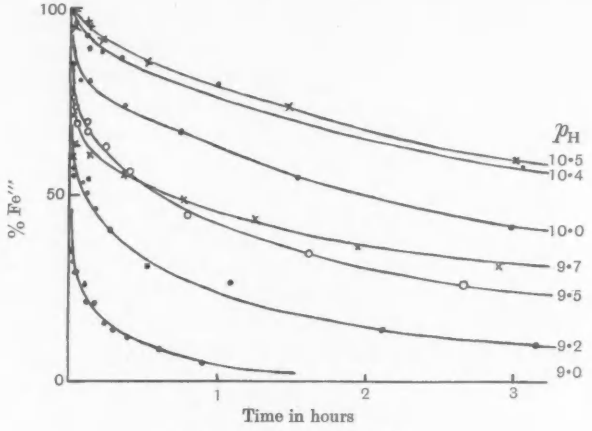


Fig. 1. Rate of autoreduction.
0·05 *M* buffer. 0·01 *M* RSH. 0·0018 *M* Fe'''.

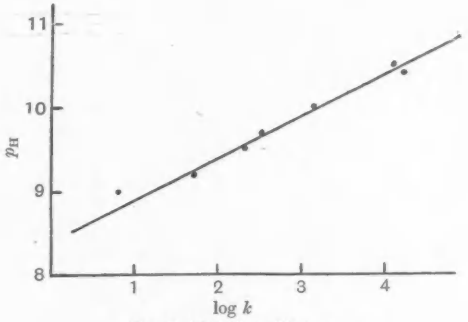


Fig. 2. Relation of k to p_H.
Continuous line is: $\log k = 16{\cdot}8 + \log [H^+]^2$.

much more rapid that it has not yet been possible to submit these to electrode analysis.

Within the titration range in which drifts were absent equilibrium potentials were always rapidly attained, remaining constant for long periods although adjusting themselves to changes in the system in a few minutes. The potentials of gold-plated platinum and bright platinum electrodes were usually within

a few tenths of a millivolt of one another. When the "poising" of the system was poor, however, there was some tendency for platinum electrodes to be displaced several millivolts negative to the gold electrodes. Since the latter exhibited the more consistent behaviour throughout, the data for these only will be reported.

A series of titrations, by both methods, was conducted at room temperature. They all conformed accurately to the rational equation (cf. (2)) for a ferrous-ferric system involving one equivalent of hydrogen. Since, however, subsequent work was standardised for a temperature of 30°, the results of these experiments will not be further utilised. In Table II the data for one titration at this temperature are recorded, the observed potentials being compared with those calculated from the equation

$$E_h = E_1 - 0{\cdot}0601 \log \frac{[Fe'']}{[Fe''']} \qquad \text{.....(2).}$$

It is of interest to note that the values of E_1 obtained at room temperature were not far removed from those found at 30°. It would seem that no large temperature correction is involved in the electrode equilibria. In passing, it should be pointed out that the agreement between the titrations with ferric iron and with hydrosulphite is the final justification for the use of the latter in the titration of ferrithiol.

Table II. *Titration at constant* p_H.

Initial system. {50 cc. 0·1 *M* carbonate buffer containing 0·030 *M* thioglycollic acid.
{4·80 cc. 0·0212 *M* ferrous sulphate in 0·01 *M* HCl.
Titrated with 0·0208 *M* ferric chloride in 0·01 *M* HCl.
$E_1 = -0{\cdot}3527$. $p_H = 10{\cdot}32$. 30°.

Titre	$0{\cdot}0601 \log \frac{[Fe']}{[Fe''']}$	E_h Calculated	E_h Observed
0·20	+0·0850	−0·4377	−0·4472
0·31	0·0715	0·4242	0·4256
0·48	0·0600	0·4127	0·4140
0·62	0·0533	0·4060	0·4056
0·95	0·0422	0·3949	0·3951
1·32	0·0337	0·3864	0·3861
1·91	0·0239	0·3766	0·3764
2·71	0·0147	0·3674	0·3676
3·71	0·0065	0·3592	0·3589
4·75	+0·0001	0·3528	0·3535
5·74	−0·0048	0·3479	0·3484
6·76	−0·0091	0·3436	0·3436

Influence of $[H^+]$.

When, in a system containing a fixed ratio of ferrous and ferric iron, the concentration of RSH or the p_H was varied, it was found that the potential was also a function of each of these variables. The limits of the experimental p_H range were approximately 8 to 10·5. The reasons for these narrow limits have already been given. Within this range the potential was found to be governed accurately by the relation

$$E_h = E_2 + 0{\cdot}0601 \log (k_a + [H^+]) \qquad \text{.....(3).}$$

The data from two experiments illustrating this relation are found in Table III. Since the changes in p_H were effected titrimetrically they involved small progressive changes in [S′]. An appropriate correction for this will be defined in a later section and is included in the table for the sake of precision.

Table III. *Relation of E_h to p_H.*

Exp. 1. Initial system. {50 cc. 0·05 *M* boric acid—KCl containing 0·0091 *M* thioglycollic acid. 1·2 cc. 0·021 *M* ferrous-ferric ions.

Titrated with 0·2 *M* sodium hydroxide at 30°.

$E_0 = +0{\cdot}1460$. $0{\cdot}0601 \log \frac{[Fe'']}{[Fe''']} = +0{\cdot}0381$. $K''' = 4 \times 10^{-3}$. $k_a = 8 \times 10^{-11}$ [Cannan and Knight, 1927].

Titre	$[S'] \times 10^3$	p_H	$0{\cdot}0601 \log (k_a + [H^+])$	$0{\cdot}0601 \log ([S'] + K''')$	E_h Calculated	E_h Observed
4·60	6·0	8·277	−0·4962	−0·1201	−0·2682	−0·2684
5·43	6·0	8·453	0·5067	0·1203	0·2785	0·2789
6·71	5·9	8·688	0·5203	0·1205	0·2919	0·2913
7·94	5·8	8·889	0·5318	0·1208	0·3031	0·3026
9·55	5·6	9·087	0·5428	0·1213	0·3136	0·3132
11·01	5·4	9·282	0·5533	0·1218	0·3236	0·3236
12·25	5·3	9·472	0·5629	0·1220	0·3330	0·3330
13·50	5·2	9·693	0·5730	0·1223	0·3428	0·3419
			0·25 cc. Fe''' added, $0{\cdot}0601 \log \frac{[Fe'']}{[Fe''']} = +0{\cdot}0185$.			
—	4·7	—	0·5730	0·1239	0·3216	0·3204
			0·50 cc. Fe''' added, $0{\cdot}0601 \log \frac{[Fe'']}{[Fe''']} = +0{\cdot}0010$.			
—	3·7	—	0·5730	0·1270	0·3010	0·3004

Exp. 2. Initial system. {25 cc. 0·044 *M* thioglycollic acid. 1 cc. 0·021 *M* ferrous-ferric ions. 10 cc. 0·2 *M* sodium hydroxide.

Titrated with 0·2 *M* hydrochloric acid.

$$0{\cdot}0601 \log \frac{[Fe'']}{[Fe''']} = +0{\cdot}0302.$$

Titre	$[S'] \times 10^3$	p_H	$0{\cdot}0601 \log (k + [H^+])$	$0{\cdot}0601 \log ([S'] + K''')$	E_h Calculated	E_h Observed
0·00	27·6	10·633	−0·5993	−0·0902	(−0·3933)	−0·3911
0·52	27·3	10·440	0·5962	0·0905	0·3899	0·3899
1·00	26·9	10·270	0·5925	0·0908	0·3859	0·3859
1·50	26·5	10·093	0·5877	0·0910	0·3809	0·3815
1·99	26·2	9·930	0·5823	0·0913	0·3752	0·3760
2·51	25·8	9·726	0·5744	0·0916	0·3670	0·3673
3·00	25·5	9·467	0·5625	0·0920	0·3547	0·3539

Influence of the concentration of RSH.

In a rigid analysis of the relation of the concentration of RSH to the potentials the function employed should be the concentration of RSH actually existing as free molecules (or ions) in the system [S′], since it is this function which must determine the degree of association of the active complexes. The value of [S′] would be obtained from [S] by subtraction of the concentration of RSH bound in the complexes. But, until the stoichiometric relations of complex formation could be presumed it was not possible to make the calculation. As a first approximation, therefore, we may be content to determine the variation of potential with [S].

Two methods were followed. In one, the total concentration of iron in all forms was varied simultaneously with [S]. In the other, the latter only was subjected to wide variation. Since the two methods indicated the same relation of E_h to [S] we may conclude that the potential is not any important function of the total concentration of iron within the experimental limits of 0·002 to 0·0001 M. The results are summarised graphically in Fig. 3.

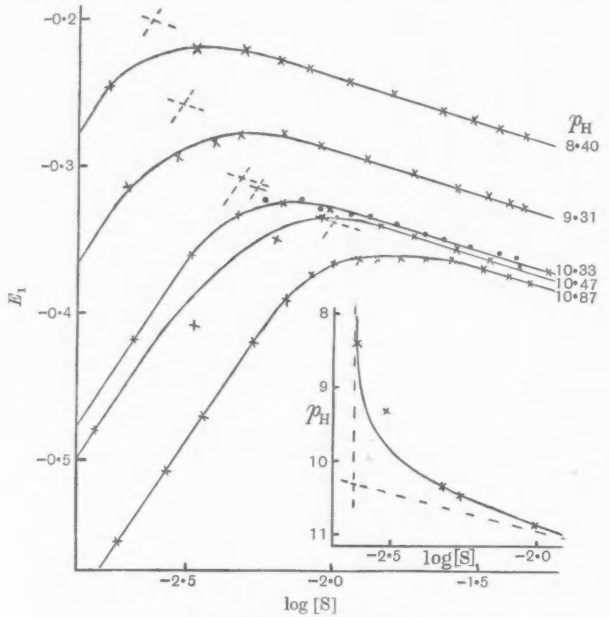


Fig. 3. Relation of E_1 to [S].
Inset: Relation to p_H of the points of intersection of the two slopes.

The interpretation of the complex relation of E_1 to [S] which is displayed in the figure is facilitated by a description of the visible changes in the system accompanying the tracing of one of the curves. As [S] is diminished the potential first follows the "0·06 slope" represented by the right-hand arm of each curve. Within the range of [S] covered by this section of the curve there is no diminution in the intensity of the red colour greater than that which can be attributed to the accompanying dilution. Consequently—if the assumption be permitted that the red colour is the active ferrithiol—the amount of oxidant is not undergoing change. The reasonable inference, therefore, since this section of the curve is defined by

$$E_h = E_3 - 0{\cdot}0601 \log [S] \qquad \text{.....(4)}$$

is that the concentration of active ferrothiol is proportional to [S]. That is to

say, ferrothiol must be substantially dissociated to the extent of one molecule of RSH per atom of iron.

As [S] is further diminished a point is reached at which a change in direction of the curve is inaugurated. The E_1 : [S] relation changes sign and, in experiments which are at favourable p_H values, ultimately attains a slope which may be described fairly accurately by

$$E_h = E_4 + 0{\cdot}0601 \log [S]^5 \qquad \text{.....(5).}$$

Accompanying this inflection in each curve there is an abrupt diminution in intensity of colour. A diffuse precipitate appears with the change in sign of the curve and almost all colour has disappeared by the time the full slope of the left-hand arm is attained. Now, to attempt the potentiometric determination of equilibrium conditions in a system containing a solid phase is always a hazardous undertaking. The observations relating to these lower ranges of [*S*] have not, therefore, the same validity as those corresponding to the systems in which no precipitate was present. Nevertheless, the potentials do represent equilibria in so far as they are definitely balanced potentials which do not vary importantly with time. Moreover, the curves are reversible in the sense that they display the same relations to [S] when traced by the progressive dilution of a concentrated iron-thiol system as by titration of a dilute solution of iron-thiol with RSH. We are satisfied that equation (5) does, in fact, correctly reflect the changes in [S] which accompany the dissociation of ferrithiol, although ignorance of the complex phase relations of iron in alkaline buffer solutions prohibits any attempt to embrace this equation in a general electrode equation. Combining the influence of equations (4) and (5) (both being operative at the lower values of [S]) we arrive at the conclusion that the concentration of active ferrithiol varies with $[S]^6$. This may be taken to confirm the suggestion that the composition of ferrithiol and, by inference, of ferrothiol, is determined by a co-ordination number of six.

Two other points may be observed in Fig. 3. The displacement of the right-hand arm of the curve with p_H is merely the graphic expression of the relation which has been established in Table III and is defined by equation (3). The displacement of the left-hand arm is not capable of such precise definition. Its significance, moreover, is confused by the unknown phase relations of iron which are involved in it. But it will be seen that this section of the curve moves with p_H towards more negative potentials at a rate greater than does the other arm. At the same time it encroaches more and more on higher values of [S]. If, as is suggested, this part of the curve betrays the influence of [S] upon the dissociation of active ferrithiol, then the tendency for the latter process must increase as the p_H rises. These varied effects of p_H are, probably, open to several interpretations, but it seems that the one which best accommodates them all is that it is the undissociated thiol molecule whose concentration determines the concentration of ferrithiol and of ferrothiol. This conclusion may be supported by the curve inset in Fig. 3. Approximate determinations of the points of intersection of the two slopes of each curve

were made graphically. The values of these points on the log [S] ordinate were then plotted against p_H. The curve resulting can be shown to be determined by a function of $k_a + [H^+]$.

If we accept the suggestions which have been advanced above as to the constitution of the complexes present in the experimental systems calculation can, then, be made of [S′]. It will later be made clear that $5\,[[Fe''] + [Fe'''']]$ is a fairly close approximation to the concentration of bound RSH. We then have

$$[S] = [S'] + 5\,[[Fe''] + [Fe'''']] \qquad \text{.....(6).}$$

If, now, [S′] replace [S] in Fig. 3 a relation is disclosed whose influence was not manifested by the less rigid treatment. Two curves in Fig. 3 are reproduced in Fig. 4 with this alteration. The difference between the relation to [S] and to [S′] can be reconciled by the equation

$$E_h = E_5 - 0{\cdot}0601 \log (K''' + [S']),$$

K′′′ being a constant with a value of 4×10^{-3}.

In Table IV this equation is applied to the calculation of theoretical potentials for comparison with those actually observed. The agreement is highly satisfactory.

Table IV. *Relation of E_h to* [S′].

Initial system. { 25 cc. boric acid—KCl buffer. 1 cc. 0·090 *M* thioglycollic acid. 1 cc. 0·021 *M* ferrous-ferric ions.

Titrated with 0·090 *M* thioglycollic acid at 30°.

$E_0 = +0{\cdot}1460$. $0{\cdot}0601 \log \frac{[Fe'']}{[Fe''']} = +0{\cdot}0473$. $K''' = 4 \times 10^{-3}$. $k_a = 8 \times 10^{-11}$.

Titre	p_H	$[S'] \times 10^3$	$0{\cdot}0601 \log ([S'] + K''')$	$0{\cdot}0601 \log (k_a + [H^+])$	E_h Calculated	E_h Observed
0·50	8·404	0·9	−0·1385	−0·5035	−0·2663	−0·2667
1·00	—	2·4	0·1317	0·5036	0·2732	0·2742
1·55	—	4·2	0·1254	0·5037	0·2796	0·2800
2·70	8·407	7·4	0·1168	0·5038	0·2883	0·2885
4·50	—	12·1	0·1078	0·5040	0·2975	0·2970
8·00	8·413	19·9	0·0975	0·5042	0·3080	0·3076
11·50	—	26·3	0·0913	0·5044	0·3144	0·3144
16·50	8·420	33·6	0·0856	0·5046	0·3203	0·3204
24·00	8·423	41·9	0·0810	0·5048	0·3251	0·3249

The value of K′′′ has been confirmed by all available data on systems not much more alkaline than p_H 9·5. Its physical significance is uncertain. We may tentatively regard it as the primary dissociation constant of ferrithiol controlling the degree of dissociation of the first RSH molecule in the complex. As such its value on the [S′] scale should be a function of k_a and should increase rapidly above p_H 10. Unfortunately, the influence of K′′′ is not clearly manifested above p_H 10, owing to the fact that, as we have seen, the [S] range of complete instability of ferrithiol encroaches more upon higher values of [S] as p_H is increased. Above p_H 10 the separate effect of K′′′ is submerged in the more general dissociation.

The several variables which have been disclosed may now be assembled in a general electrode equation which will read

$$E_h = E_0 - 0{\cdot}0601 \log \frac{[Fe'']}{[Fe''']} - 0{\cdot}0601 \log \frac{[S']+[K''']}{k_a+[H^+]} \quad \ldots\ldots(7).$$

This is applicable only to systems containing no solid phase of iron, within the p_H limits 8–10·5 and within the limits of [S'] which vary with p_H but are roughly 0·005 to 0·1 *M*.

It is this equation which has been employed for the calculation of theoretical potentials in Tables II–IV. The least satisfactory part of this has been the quantitative evaluation of E_0. Normally this would have been established with precision from the data of a titration at constant p_H and [S]. It was, however, pointed out at the beginning of this section that such titrations could

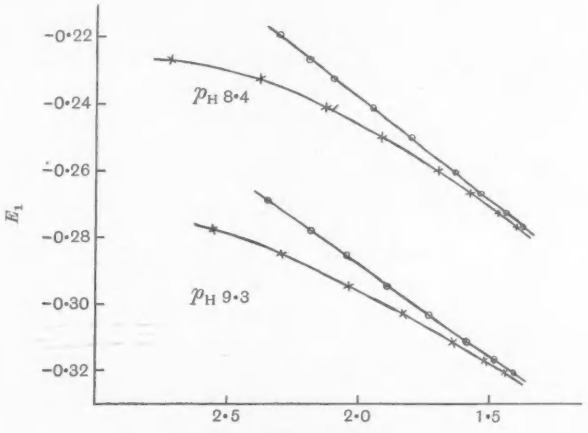


Fig. 4. Influence of K'''.
⊙ = log [S] × = log [S']

only be conducted in the neighbourhood of p_H 10. Now, as has just been shown, it is at such a p_H that the absolute magnitude of [S'] + K''' is uncertain. The derivation of E_0 from experiments below p_H 9 in which a predetermined ratio of ferrous and ferric ions was added to a RSH solution is equally unsafe. This is due to the difficulty of ensuring that a given ferrous-ferric ratio retains that ratio precisely when added to a deaerated solution of RSH. Traces of oxygen introduced with the burette tip or momentary local concentration effects leading to local autoreduction are two factors liable to modify an experimental ratio. The procedure which has been adopted has been to assign to E_0 the value 0·1460 because this fits closely the majority of the data. Such observations as disagreed indicated lower values, the lowest being 0·1380. With E_0 fixed experimental values of $\log \frac{[Fe'']}{[Fe''']}$ were then adjusted to give the best alignment with E_0. The magnitude of this correction was, generally, small.

In conclusion, allusion must be made to one point in the electrode analysis. It is surprising that in a series of experimental systems it should have been possible to replace the rational term $[S'] + K'''$ by the empirical term $[S']$ without serious distortion of the data. This was of the nature of a fortunate coincidence. It happened that within those rather narrow ranges of [S] in which K''' was a significant term the experimental values of $5\,[[Fe''] + [Fe''']]$ approximated closely to 4×10^{-3}. The small differences between [S] and $[S'] + K'''$ were still less evident in the logarithmic terms.

Chemical description of the system.

It is always dangerous to translate thermodynamic relations into chemical structures. Yet if we are to define systems such as iron-cysteine, iron-glutathione, etc., which are not amenable to extensive electrometric analysis, some attempt to generalise the system in broad chemical terms must be made.

The detailed analysis which has been presented is consistent with the following scheme. The reversible potentials are controlled by the oxidation-reduction equilibrium:

$$\underset{\text{(ferrothiol)}}{[Fe^{II}(RSH)_6]^{++}} = \underset{\text{(ferrithiol)}}{[Fe^{III}(RSH)_6]^{+++}} + e.$$

The concentrations of these active reactants are determined by the equilibria:

$$[Fe^{II}(RSH)_6]^{++} = [Fe^{II}(RSH)_5X]^{++} + RSH$$

Dissociation constant, K''

$$[Fe^{III}(RSH)_6]^{+++} = [Fe^{III}(RSH)_5X]^{+++} + RSH$$

Dissociation constant, K'''

$$[Fe^{III}(RSH)_6]^{+++} + H_2O = [Fe^{III}(RSH)_6]^{++}OH + H^+$$

Hydrion dissociation constant, k_b

and

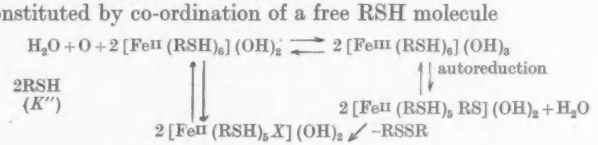
$$RSH = RS^- + H^+$$

Hydrion dissociation constant, k_a

The further dissociation of the complexes with liberation of more RSH molecules, and the remaining hydrion dissociations of the complex cations, may be ignored since they do not influence the behaviour of the system within the experimental ranges of $[S']$ and p_H within which equation (7) holds. The nature of X, the component which has been included to complete the co-ordination ring, is unknown. It might be a molecule of water by analogy with the aquopentacyano-ferroates of Hofmann [1900], or a carboxyl group of one of the co-ordinated RSH molecules. In any case, this hypothesis of a potential vacancy in the co-ordination ring of iron-thiol complexes allies them closely with a number of other co-ordinated iron compounds [cf. Manchot, 1912], and even, on the basis of Conant's views [1923], with haemoglobin and related substances. Possibly, the peculiar relations of RSH with carbon monoxide, oxygen, hydrocyanic acid, hydrogen sulphide, and perhaps, the "fixed SH" of tissues [Hopkins and Dixon, 1922], may be due to preliminary co-ordination of these molecules in the vacant position of ferrothiol.

The spontaneous autoreduction of ferrothiol may be described as an intramolecular process in which an electron passes from a co-ordinated RSH

molecule to the iron atom. The free radicle of RSSR which results then dissociates from the complex and polymerises to RSSR. The ferrous complex is then reconstituted by co-ordination of a free RSH molecule



This complex set of equilibria is probably less complex than the situation which obtains in the system RSH—Fe. It does, however, give a plausible description of the catalytic influence of iron upon the oxidation of RSH, and it does not appear necessary as yet to complicate the situation by the consideration of further variables.

It remains to add that the theoretical equation which may be derived for the hypothetical system

$$(\text{ferrothiol})^{++} = (\text{ferrithiol})^{+++} + e$$

may be reduced to equation (7) provided that we make certain reasonable assumptions. These are, that K″ is very great compared with [S′], and that both complexes are completely dissociated as bivalent cations within the experimental range of p_H. The former assumption is implicit in the treatment which has been accorded the electrode data. The latter receives support from the colour change of ferrithiol in the neighbourhood of p_H 5. This may correspond with the third basic constant of the oxidant.

Reference has been made to the recent studies of the complexes formed between cysteine and cobalt [Michaelis and Barron, 1929, 2, and Michaelis and Yamaguchi, 1929]. It appears that cobalt forms with cysteine a cobaltous complex which is almost colourless in dilute solution. This substance is very readily oxidised to a reddish brown product, in which, as is definitely indicated by the evidence of these authors, RSH and Co are present in a ratio of 3 : 1. Since the oxidation involves two equivalents of hydrogen for each atom of cobalt, Michaelis and Yamaguchi believe that, in the coloured product, not only has cobalt been oxidised to the cobaltic state but also that one of the combined RSH molecules has lost one equivalent of hydrogen. If we understand their argument, the process may be represented as follows:

$$\left[Co^{II}\left(\begin{matrix}OOC\\S\end{matrix}R\right)_3\right]H_4 = \left[Co^{III}\left(\begin{matrix}OOC\\S\end{matrix}R\right)_3\right]H_2 + 2H$$

or

$$\left[Co^{II}\left(\begin{matrix}OOC\\HS\end{matrix}\!\!>R\right)_3\right]H = \left[Co^{III}\left(\begin{matrix}OOC\\HS\end{matrix}\!\!>R\right)_2\left(\begin{matrix}OOC\\S\end{matrix}\!\!>R\right)\right] + 2H$$

according to whether the undissociated RSH or the thiol anion be regarded as the group co-ordinated.

Michaelis and Barron have suggested that their oxidised cobalt complex is comparable to the coloured ferrithiol complex, and suppose that the difference is merely that the co-ordination complex "which is intermediary, unstable,

and inaccessible therefore for chemical analysis in the case of iron, is stable and suitable for chemical analysis in the case of cobalt." We believe the two compounds to be distinct in respect of both the number of co-ordinated thiol groups and of the number of equivalents of oxygen necessary to create them from the reduced complexes. Our titrations indicate unequivocally that each atom of ferrous iron required only one equivalent to produce the active coloured oxidant. But apart from these distinctions, there are many points in common in the structural formulations which Michaelis and Yamaguchi have adopted for cobalt-cysteine and which we have suggested for iron-thioglycollic acid.

One point in the work of Michaelis and Barron requires further discussion. They showed that systems of cobalt and cysteine developed intensely negative potentials at an inert electrode. The reproducibility of these potentials appears however to have been poor, and no detailed study of them was made. The oxidation of the cobaltous-cysteine was, however, followed electrometrically, and as a result the authors arrive at the conclusion that the progress of the reaction varies with the nature of the oxidising agent. When ferricyanide was employed there was evidence of the removal of the two equivalents separately. In other words, there was evidence of a stable intermediate. When, on the other hand, indophenol was employed as oxidising agent there was no evidence of this stepwise oxidation to the cobaltic dithiol product. The authors suggest that cobaltous cysteine may be oxidised in two individual steps by ferricyanide, because the latter only requires a single equivalent of hydrogen per molecule on reduction. Indophenol, they argue, can only oxidise by accepting a pair of equivalents owing to the quinonoid structure of the molecule.

To this conclusion we cannot subscribe. The distinction between the progress of the reaction in the two cases which is indicated by the dissimilar electrode titration curves, is, after all, merely a thermodynamic abstraction. It can carry no definite implications as to the molecular processes involved. If the titration curves are significant, they rest upon the assumption that the sole purpose of the oxidising agent is to alter the ratio of the oxidant to reductant in the system. In that case there is no thermodynamic basis for distinguishing between the two oxidising agents. Therefore a difference between the types of curve traced in the two cases must be attributed to secondary reactions induced by the presence of one or other of the titrating agents. It is appropriate, therefore, to question whether ferricyanide was a happy choice of reagent for titrating, without complication, the co-ordination complexes of cobalt. Be that as it may, it must be pointed out that the systems which Michaelis and Barron titrated with ferricyanide and indophenol respectively were very different. In the indophenol titrations the whole of the cobalt was present as the cysteine complex, but in the titration with ferricyanide there was only sufficient cysteine present to co-ordinate one-half of the cobalt. Such a difference would be expected to have an important effect on the titration curve.

THE ELECTRODE BEHAVIOUR OF RSH WITHOUT ADDED IRON.

Reference has already been made to the anomalous electrode behaviour of solutions of "pure" thiol compounds. A great deal of attention has been devoted to this problem since the first observations of Dixon and Quastel [1923]. These workers found that inert electrodes of gold or platinum in solutions of cysteine or glutathione developed potentials which were approximately defined by

$$E_h = E_0 - 0{\cdot}06 \log \frac{[S]}{[H^+]}.$$

The least satisfactory character of this equation was, as has already been described, the wide variation in the absolute magnitude of E_0 in different experiments. Dixon [1927] extended the equation to the behaviour of the mercury electrode. He made the plausible suggestion, though on quite inadequate data, that a more comprehensive relation is given by

$$E_h = E_0 - 0{\cdot}06 \log \frac{[S]}{[k_a + [H^+]]}.$$

As a matter of fact, our own observations, which need not be reported in detail for the present argument, on the mercury-thioglycollic acid electrode have established the validity of the modified equation with some precision. The further study of the problem has been greatly advanced by the work of Michaelis and Flexner [1928] who have established the conditions of technique appropriate to the most reproducible results.

The fundamental peculiarity of the thiol electrode is that the potentials are independent of the concentration of RSSR; that is to say, they are not a function of the concentration of the presumed oxidant of RSH. Dixon and Quastel made the suggestion that this might be due to the fact that the immediate oxidant which was in equilibrium with RSH was a hypothetical intermediate whose concentration was independent of RSSR. In a later paper, however, Dixon [1927] abandoned this difficult assumption for a general hypothesis of electrode mechanism which, based upon kinetic considerations of hydrogen transport and Wieland's theory, was avowedly designed to explain the anomalies of the thiol electrode. The theory of Dixon has not been found acceptable by other students of the problem, and, since it was a kinetic theory unsupported by kinetic evidence, it need not be further discussed here. Michaelis and Flexner were unable to arrive at any satisfactory explanation of the thiol electrode.

The results which have been reported in the present paper upon the electromotive activity of the thiol-iron complexes naturally raise the question whether these may not be responsible for the behaviour of so-called pure solutions of RSH. No confident answer can be given. There is a superficial resemblance between the electrode equation for the iron-thiol system and the equation of Dixon. But the highly complex relation of the iron system to the concentration of RSH indicates that the behaviour of the two electrodes is very different. It seems quite clear that the thiol electrode does not behave

merely as a system in which the ratio $\frac{[Fe'']}{[Fe''']}$ has been held constant by the experimental technique employed. At the same time, it is not possible to deny that the influence of the dissociation of the complexes upon the potentials might be neutralised by changes in the ferrous-ferric ratio taking place even in the absence of oxygen. This situation might well arise as the result of experimental modifications of the system reacting, through the various equilibria of complex formation, upon the velocity of autoreduction. However this may be, there seems little advantage at present in adopting an explanation of the thiol electrode which is no less artificial and abstract than that originally suggested by Dixon and Quastel.

In a future communication we hope to return to the question of the thiol electrode both from the electrometric and kinetic points of view. The object will be to suggest the advantage of attributing to the system reversible equilibria not conditioned by the presence of casual traces of one metal or another. With such a system ferro-ferrithiol, thiol-mercury thiol [cf. Barron, Flexner and Michaelis, 1929] or any other reversible system present may be considered to be in simultaneous equilibrium. It then becomes merely a matter of stoichiometric or kinetic predominance to which system we choose to assign responsibility for the phenomena.

Experimental detail.

Autoreduction of ferrithiol. A solution of thioglycollic acid in a deaerated carbonate buffer (0·1 *M*) of suitable p_H was prepared and stored in the reservoir of a burette protected from oxygen. The p_H of an aliquot portion was determined by means of the hydrogen electrode. A known volume of the remainder was transferred to another vessel under pure nitrogen, a known amount of standard deaerated ferric chloride solution was added at zero time and the mixture returned to the reservoir. At suitable intervals, samples were removed and titrated with a standard solution of sodium hydrosulphite. The most careful protection of the reaction mixture from all traces of oxygen during preparation, storage and titration was essential for reliable results. In those cases in which it was desired to make determinations after only short reaction times, individual mixtures of iron and RSH were made in the actual titration vessel and titrated after the desired interval. The concentrations of the components were, approximately, 0·05 *M* buffer, 0·01 *M* RSH and 0·0018 *M* iron. Owing to the difficulty of conducting such operations within a thermostat, the experiments were carried out at room temperature (13–16°).

The sodium hydrosulphite was standardised by means of a standard solution of ferric iron prepared from ferrous ammonium sulphate and its equivalent of potassium permanganate. Methylene blue served as indicator. In the titration of ferrithiol no added indicator was necessary. The justification for the employment of hydrosulphite to titrate ferrithiol has been given in the discussion of the electrode studies. It may be added that the fall in titration

value was parallel with the decay of the intensity of the red colour. Moreover, in those cases in which the rate of the reaction was slow, the initial titrations were equal to the total amount of ferric iron originally added. The only reaction likely to confuse the titration would be the oxidation of ferrothiol or hydrosulphite by RSSR. Control experiments established the absence of these reactions under the experimental conditions.

Electrode titration at constant p_H *and constant* $[S]$. Two methods were followed. 1. A standard solution of ferric chloride was added to a buffered deaerated solution of RSH in the electrode vessel. Autoreduction was allowed to proceed until its velocity, as indicated by the absence of further potential drifts, became insignificant. The titration of the residual ferrithiol with sodium hydrosulphite was then commenced. The end-point of this titration was identified with the disappearance of the last trace of red colour. With this determined and a knowledge of the amount of ferric iron originally added the ratio $\frac{[Fe'']}{[Fe''']}$ at each step in the titration could be calculated. 2. In the second method a known volume of a standard solution of ferrous sulphate was added to a buffered, deaerated solution of RSH. The mixture was then titrated with ferric chloride. Equilibrium potentials were always attained within 2–5 minutes of each change in the system and remained constant for long periods. With the progress of the titration and the consequent increase in $\frac{[Fe''']}{[Fe'']} = \frac{x}{a-x}$, a point was ultimately reached at which significant negative drifts of potentials suddenly became apparent. These were undoubtedly due to the magnification of the rate of autoreduction as the ratio of ferrithiol to ferrothiol increased. The first appearance of these drifts terminated the titration since the nature of the velocity curve discouraged attempts to extend the titration by a series of hurried observations.

Relation of E_h *to* p_H. A 0·01 M solution of RSH in 0·05 M boric acid buffer was adjusted to a p_H of about 8 and deaerated. A mixture of ferrous and ferric ions in known proportions was added and the solution then titrated with deaerated sodium hydroxide. Alternatively, a solution adjusted to about p_H 11 was titrated with hydrochloric acid. Periodic determinations of the equilibrium potentials were made. The corresponding changes in p_H were determined by means of a hydrogen electrode titration in which all conditions, except the presence of the iron, were duplicated.

Relation of E_h *to* $[S]$. Two methods were followed. 1. To a dilute buffered solution of RSH was added a known ratio of ferrous and ferric ions. The mixture was then titrated with a concentrated solution of RSH identically buffered. Equilibrium potentials were determined after each addition. Incidental minor changes in p_H were controlled by a duplicate titration at the hydrogen electrode (the iron only being omitted from the system). 2. A concentrated (0·1 M) buffered solution of RSH was progressively diluted with the pure buffer solution and the changes in potential determined. Changes in p_H were not, in this case, controlled and this may account for the

small discrepancies between observed and calculated potentials during the first three dilutions. This would arise were the pure buffer and the buffered RSH not identical in p_H.

Summary.

1. Both ferrous and ferric ions form soluble complexes with certain thiol acids. The ferrithiol complex suffers spontaneous autoreduction to the corresponding ferrothiol. A preliminary study has been made of the rate of this reaction and of its relation to p_H. The rate of autoxidation of ferrothiol has been considered.

2. Ferrithiol-ferrothiol constitutes a reversible oxidation-reduction system which is electromotively active. The equilibrium potentials of the system have been determined and an electrode equation has been established which defines the electrode behaviour within a limited range of p_H and concentration of thiol acid.

3. Arising out of these studies certain suggestions have been made as to the nature of the complexes. These have been elaborated to a tentative structural description of the various equilibria of importance in the system thiol acid-iron.

4. The purpose of these studies is to provide a quantitative basis for the interpretation of the catalytic influence of iron on the thiol-disulphide system and the anomalous electrode activity of thiol compounds.

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CXXXIX. THE DETERMINATION OF SOAP IN BLOOD.

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(*Received September 17th, 1929.*)

It has long been known that the fatty acids of the blood occur not only in combination as fats and cholesteryl esters, but also in small amounts either free or as simple soaps. Whether or no soaps exist as such, is problematical, since it appears probable that such substances, under the conditions present in blood, would undergo salt hydrolysis, with consequent formation of free fatty acid. It is, of course, this salt hydrolysis which renders impossible the titration of free fatty acid with alkali in aqueous solution. In the presence of alcohol, however, the hydrolysis does not take place, and titration is possible. When, therefore, blood, which is slightly alkaline, is poured into a large excess of alcohol, soap is present, whether it existed in the blood previously as such or as free fatty acid.

Exact figures as to the total amount of soap present and also suitable methods for determining this amount have not hitherto been readily available. Yet some relatively simple method of estimation might prove of value in assisting to complete the picture of the changes occurring in the organism during fat metabolism. The usual methods for the estimation of soaps in biological fluids involve extraction with some solvent in which the soap is especially soluble and the oxidimetric titration of the fat radicle [Bang, 1918; Condorelli, 1922].

Such methods are open to considerable objection since it is impossible to get a complete separation of fats from soaps, by means of selective solvents—in none of the usual fat solvents is either of these substances absolutely insoluble—and the amounts of fat and soap present in a biological fluid may be very small indeed. Thus there is always the possibility that some of the substance finally oxidised during the titration may not necessarily be soap and the results obtained must therefore be regarded as qualitative rather than quantitative, or at best, roughly quantitative through compensation of errors. Gravimetric estimation [Lemelland, 1921] is open to the same objection although larger amounts of material must be used for analysis. A satisfactory method for the determination of soap must therefore avoid dependence on selective solubility, and it would seem, therefore, that the point of attack should be, not the fatty acid portion of the molecule which is common to soap and fat, but the metallic part which is peculiar to the soap. Were it then

possible to titrate the base combined with the fatty acid without affecting that combined with other acids in the alcohol-ether extract we should have a relatively simple means of estimating the soap content of the blood.

We therefore carried out a series of experiments to find with what degree of accuracy it was possible to determine small amounts of soap, using such volumes of fluid as could be titrated with the Rehberg burette. 0·5 cc. of alcoholic solution of pure soap was evaporated to dryness in a hard glass test-tube (6 by 1·5 cm.), 0·5 cc. $N/350$ H_2SO_4 added and the mixture titrated with $N/70$ NaOH with 0·2 cc. bromocresol purple as indicator. The end-point was taken at the first colour change. The difference between a blank titration with 0·5 cc. $N/350$ H_2SO_4 and the titration in the presence of soaps gave the amount of alkali combined with fatty acid. Various indicators other than bromocresol purple were tried but offered no advantages.

In the course of these experiments we found it essential to keep the amount of alcohol at a minimum—hence the evaporation to dryness of the soap solution. Besides interfering with the sharpness of the end-point even in the control titrations, excess of alcohol dissolved the fatty acid, which, in solution, was sufficiently dissociated to prevent the titration of the mineral acid alone. In the absence of alcohol, and with the indicator chosen, the end-point is sufficiently sharp, and occurs when the free mineral acid has been completely neutralised and the fatty acid remains untouched.

A further source of error was the extraction of estimatable amounts of alkali from the glassware during the evaporation of the alcoholic soap solution. Since this error was appreciable even during the evaporation of such small amounts, and was, moreover, variable, it would obviously have proved fatal to the method when applied to blood with the consequent concentration of larger volumes. We found it possible, however, to eliminate it entirely by the use of hard glass test-tubes (clear silica tubes, of course, would be ideal) with silica chips to prevent bumping during the evaporation. In applying the method to blood later we used silica beakers for the evaporation of the alcohol-ether extract. The amount of heating involved in the preparation of the alcohol-ether extract did not, we found, introduce any appreciable error. A specimen series of analyses is given in Table I.

Table I.

cc. soap	cc. $N/350$ H_2SO_4	cc. NaOH (0·0149 N)	cc. NaOH equivalent to fatty acid	Sodium oleate (mg.) Found	Present	% error
0·0	0·5	0·0890	—	—	—	—
0·0	0·5	0·0890	—	—	—	—
0·1	0·5	0·0685	0·0205	0·0927	0·095	−3·0
0·2	0·5	0·0463	0·0427	0·1930	0·190	+1·5
0·4	0·5	0·0075	0·0815	0·3690	0·380	−3·0

The percentage error varied from + 1·5 % to − 3 % within the range of error of measurement of the solutions to be titrated which were delivered from a graduated 1 cc. pipette.

Thus a satisfactory degree of accuracy can be obtained in the determination of small amounts of pure soap solution, the error being no greater than that involved in the measurement of small volumes of fluid.

In applying the method to blood, the usual mixture of alcohol (3 vols) and ether (1 vol.) was used for the extraction of the fatty substances. 2 cc. of blood were added drop by drop to 30 cc. of the mixture in a 50 cc. flask. The flask was shaken throughout the addition of the blood, and was then heated on the water-bath until the ether began to boil. It was then cooled and alcohol-ether was added to the 50 cc. mark. After thorough mixing, the sediment was allowed to settle. 20 cc. of the supernatant liquor were pipetted off and filtered through a small plug of ether-cleansed glass wool into a silica beaker. The plug was washed with alcohol-ether, and the washings were run into the beaker. The contents of the beaker were evaporated to dryness on the water-bath (overheating must be sedulously avoided) and the residue was thrice extracted with about 1 cc. of hot absolute alcohol. The combined extracts were evaporated to dryness in a hard glass test-tube with silica chips to prevent bumping and 0·5 cc. $N/350$ H_2SO_4 was added to the residue. The liquid was cloudy because of the presence of fats and free fatty acids and titration was impossible owing to turbidity and to adsorption of the indicator on the fat. To overcome this difficulty we added 0·5 cc. of a saturated solution of pure sodium chloride and about 1 cc. of pure light petroleum. After standing overnight in the ice-chest the liquid cleared and titration was possible. The results of several such determinations in duplicate are given in Table II.

Table II. *Determination of soap in whole blood.*

The cc. $N/70$ NaOH used represents the difference between the control titration and that of the specimen.

	Vol. of blood in extract (cc.)	cc. $N/70$ NaOH used	Mg. oleic acid per 100 cc. blood
Blood 1	1·0	0·0859	34·6
„ 1	1·0	0·0854	34·4
Blood 2	1·0	0·0661	26·6
„ 2	1·0	0·0659	26·5
Blood 3	0·4	0·0400	40·3
„ 3	0·4	0·0420	42·3

It is impossible to prove directly that the substance determined is soap, though from indirect evidence it appears highly probable that this really is the case. In the first instance, soap added to blood could be determined with reasonable accuracy (Table III).

Table III. *Determination of added soap.*

Alcoholic solution of pure sodium oleate (equivalent to 19·3 mg. oleic acid per 100 cc.) added to alcohol-ether extract of blood.

	cc. $N/70$ NaOH used	cc. $N/70$ NaOH used for added soap	Mg. oleic acid per 100 cc. soap solution
10 cc. blood extract (0·4 cc. in extract) ...	15·5	—	—
10 cc. blood extract + 0·04 cc. soap solution	35·0	19·5	19·6
10 cc. blood extract + 0·08 cc. soap solution	53·4	37·9	19·1

Further, the substance determined cannot be a salt of phospholipin, or diphosphoglyceric acid, since its concentration is greater in the plasma than in the corpuscles (Table IV).

Table IV. *Determination of soap in whole blood and plasma.*

	Mg. oleic acid per 100 cc.		Plasma. Vol. % of whole blood	Mg. oleic acid whole blood calc. from plasma content
	Whole blood	Plasma		
1. Normal	34·5	46·7	60	28·0
2. Normal	57·4	98·7	60	59·2
3. Polycythaemia	45·3	112·8	40	45·1

It cannot be a salt of lactic acid, acetoacetic acid, or hydroxybutyric acid since the sodium salts of these acids added to blood are not determined by the method (Table V). The dissociation constants of these last two acids are so similar to that of butyric acid that this statement may be taken as correct, although the experiment has not actually been carried out owing to the difficulty of obtaining these substances. Moreover, the amount of soap actually found in normal blood is greater than would be accounted for by the small amounts of "acetone bodies" known to be present.

Table V. *The non-interference of added lower fatty acid salts and of added fat.*

	Mg. oleic acid per 100 cc. blood
Blood alone	18·82
Blood + 400 mg. sodium lactate per 100 cc.	18·88
Blood alone	31·00
Blood + 400 mg. sodium butyrate per 100 cc.	30·30
Blood alone	28·60
Blood + 500 mg. tripalmitin per 100 cc. ...	28·30

Incidentally it might be remarked that such a method as this which we have described might be useful in following the activity of lipase in blood.

Summary.

A method is described for the determination of soap (or free fatty acid) in blood. For normal human blood the concentration calculated as oleic acid is 18–40 mg. per 100 cc., for plasma 46·7–98·7 mg. per 100 cc. in the specimens we have so far examined.

We wish to express our thanks to the Moray Fund of this University, a grant from which defrayed a part of the expenses of this work. One of us (C. P. S.) is in receipt of a part-time grant from the Medical Research Council.

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CXL. VITAMIN A AND CAROTENE.

II. THE VITAMIN A ACTIVITY OF RED PALM OIL CAROTENE. III. THE ABSENCE OF VITAMIN D FROM CAROTENE. IV. THE EFFECT OF VARIOUS DIETARY MODIFICATIONS UPON THE VITAMIN A ACTIVITY OF CAROTENE.

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Report to the Medical Research Council.

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(*Received November 5th, 1929.*)

II. THE VITAMIN A ACTIVITY OF RED PALM OIL CAROTENE.

In a previous communication [Moore, 1929, 1], in which the literature was quoted, confirmation was afforded to the claim of Euler, Euler and Hellstrøm [1928] that the pigment carotene, in a state of apparent purity, possessed intense vitamin A activity. In this work the carotene was derived exclusively from carrots. Collison, Hume, Smedley-MacLean and Smith [1929], on the other hand, have demonstrated the activity of carotene not only from carrots but also from the green leaves of cabbage and spinach. Since other pigments, such as xanthophyll, lycopin, α-crocetin, bixin and capsanthin have been reported by Euler, Euler and Karrer [1929, 1] to possess no growth-promoting powers[1], attention must tend to be focussed on carotene as affording some peculiar clue to the problem of vitamin A activity. Experiments by the writer [Moore, 1929, 2] have, in fact, indicated the possibility that carotene may behave *in vivo* as a precursor of the vitamin. It is therefore of interest to test the activity of carotene derived from as many different sources as possible. The present experiments demonstrate the activity of a sample of the pigment prepared from red palm oil.

Experimental.

A sample of the unsaponifiable matter of red palm oil was made available through the courtesy of Mr MacLennan of Lever Brothers, Limited. Colorimetric examination revealed the presence of considerable amounts of pigment, but when methods similar to those used in the isolation of carotene from carrot

[1] It was, however, reported by these workers that dihydro-α-crocetin possesses vitamin A activity. Confirmatory evidence of this surprising claim has not yet been put forward (see Karrer [1929]).

fat were applied it was found that no satisfactory separation of the pigment could be effected. The problem of isolating the bulk of the carotene, indeed, has not yet been solved, but tests have been carried out on a small specimen obtained in the following manner. From about 10 cc. of the crude unsaponifiable matter in chloroform solution a small fraction of brown amorphous material was removed by the addition of excess of methyl alcohol. The filtrate, after evaporation of the solvent under diminished pressure, was stored for several weeks in a refrigerating chamber. A small yield of crystals was then noticed. These were removed, washed with methyl alcohol, and recrystallised twice from chloroform by the addition of methyl alcohol. Only about 5 mg. of the pigment (M.P. 162° in air) was obtained, an amount which was obviously too small to permit of further purification. The appearance, solubility and chromogenic value of the crystals, however, all indicated that the pigment was carotene.

Colorimetric examination. As a preliminary to biological tests both the crude unsaponifiable matter and the isolated pigment (the latter dissolved in arachis oil at the concentration of 0·01 mg. per 20 mg. drop of oil) were examined both in regard to their degree of natural yellow pigmentation in chloroform solution and in regard to the depth of the blue colorations which were given with the $SbCl_3$ reagent.

Colorimetric technique. The colorimetric methods used throughout this research would seem to be liable to inaccuracies when applied indiscriminately over wide ranges of concentration. It is desirable, therefore, to describe in detail the technique used and the mode of presentation of results. Observations were carried out in a Rosenheim-Schuster tintometer fitted with the usual circular tube of 1 cm. diameter. In estimations of natural yellow pigmentation, (*a*) the given material was dissolved in chloroform and diluted with the same solvent until a reading of about five yellow units was given, (*b*) this reading was then divided by the amount of material in mg. present in 1 cc. of solution thus giving the value in yellow units calculated for 1 mg. of the material dissolved in 1 cc. of solvent and viewed in a layer 1 cm. thick (for brevity "1 cm. cube"). In evaluating the blue colorations given with the $SbCl_3$ reagent, (*a*) a known volume (calculated by trial experiments to give a reading of about five units) of a standardised chloroform solution of the material was delivered into the tintometer tube by means of a blood pipette and the volume made up with washings to 0·5 cc., (*b*) 2 cc. of the $SbCl_3$ reagent was added and a reading immediately taken, (*c*) the mean of several readings was divided by the total amount of the material present and multiplied by the total volume (2·5) in cc. thus giving the value in blue units calculated for 1 mg. of material dissolved in "1 cm. cube" of reagent. It should be noted that the values so calculated would not necessarily agree with experimental findings at the same concentration, as depth of colour does not seem to bear a linear relation to the amount of material taken. The advantage of this uniform method of expression lies rather in its simplicity, and in its convenience when it is desired

to compare the degrees of natural yellow pigmentation of materials with their activity in the $SbCl_3$ reaction.

The following data were obtained:

	Lovibond yellow units per mg. per "1 cm. cube" $CHCl_3$	Lovibond blue units (at 590 $\mu\mu$) per mg. per "1 cm. cube" of $SbCl_3$ reagent
Crude unsaponifiable fraction	75	16
Crystalline pigment	750	190

Physiological activity. Biological tests of both the crude unsaponifiable fraction and the isolated pigment were carried out, using the technique described in the preceding paper. It will be seen from the growth curves (Fig. 1) that 0·01 mg. of the pigment sufficed to restore slow growth in two rats, whereas the minimal dose of the concentrate lay at a higher level, 0·02 mg. giving no response and 0·2 mg. sufficing for irregular growth.

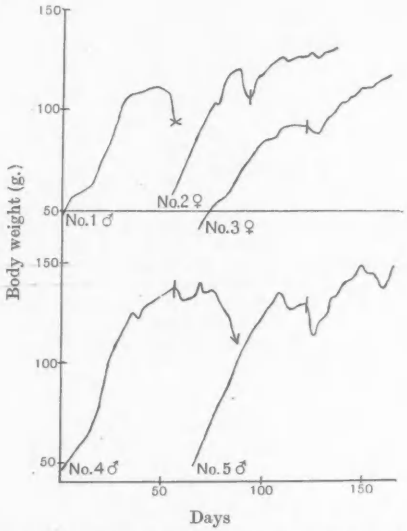

Fig. 1. The vitamin A activity of red palm oil carotene.
Rat No. 1: negative control.
Rats Nos. 2 and 3: 0·01 mg. carotene daily.
Rat No. 4: 0·02 mg. crude unsaponifiable matter.
Rat No. 5: 0·2 mg. crude unsaponifiable matter.

It may be recalled that carotene derived from carrots is of much greater activity than the fat from which it has been separated. It is noteworthy, therefore, that the concentration of activity in the crystalline pigment has now been confirmed in the case of a preparation of entirely different origin.

III. THE ABSENCE OF VITAMIN D FROM CAROTENE.

Although it was originally suggested by Euler, Euler and Hellstrøm [1928] that the failure of previous investigators to obtain evidence of the vitamin A activity of carotene might have been due to the absence of vitamin D from the test diets employed, it has more recently been reported by Euler, Euler and Karrer [1929, 2] that omission of vitamin D from diets containing carotene does not cause cessation of growth, but leads to faulty calcification. From this result it has been inferred that carotene does not possess antirachitic power[1]. It must be remarked, however, that in these experiments an ordinary complete basal diet, as opposed to a rachitogenic diet, seems to have been employed, and also that the antirachitic value of the pigment was tested only at relatively low levels. For these reasons it seemed desirable to confirm the inactivity of the pigment under more conventional conditions.

Experimental.

Young albino rats of 40–60 g. were placed upon the Steenbock rachitogenic diet No. 2965. Graded doses of carotene, dissolved in arachis oil, were administered daily from the commencement of the experiment. After 35 days the rats were killed and the rib junctions examined macroscopically. The following results were obtained:

Sex and weight (g.)	Daily supplement	Condition of ribs	Relative severity of rickets
♀ (59–114)	200 mg. arachis oil (negative control)	Arch good, no fractures or angulation. Costochondral junctions slightly enlarged. Lines slightly hazy	+
♂ (54–126)	0·0001 mg. carotene	Arch good, etc. Costochondral junctions greatly enlarged with cupping and almost complete blurring of lines	+ + +
♂ (64–143)	0·001 ,,		
♂ (45–150)	0·01 ,,		
♂ (47–132)	0·1 ,,	Arch good, etc. Costochondral junctions moderately enlarged with lines hazy	+ +
♂ (57–140)	0·75 ,,		
♀ (36–103)	1 mg. cod-liver oil concentrate (positive control)	Normal	0

The carotene, therefore, did not prevent the development of rickets when administered at a level equivalent to about 100 times the minimal dose for vitamin A[2].

IV. THE EFFECT OF VARIOUS DIETARY MODIFICATIONS UPON THE VITAMIN A ACTIVITY OF CAROTENE.

It has been suggested [Moore, 1929, 1] that the cause of the divergent results of previous workers upon the activity of carotene might lie in some

[1] The position was further complicated by the claim that vitamin A (carotene) was essential for the promotion of normal calcification even on diets considered by these authors to contain an adequate amount of vitamin D (2 mg. of irradiated arachis oil).

[2] The carotene tested in this experiment was prepared from carrots. A similar sample has subsequently been examined by Mr A. L. Bacharach, to whom my thanks are due. No vitamin D activity could be detected at 0·11 mg. by the faecal p_H method, nor at 0·045 mg. by the line test method.

unsuspected difference in biological technique rather than in the degree of purity of the various samples examined. In particular it was considered that the presence of fat in the basal diet might be of some importance. Experiments to decide this point, and also the effect of some other minor alterations in technique, have now been carried out.

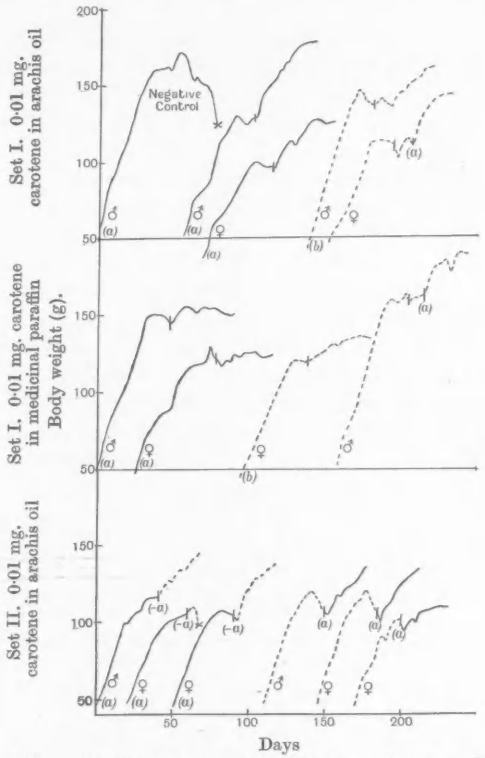

Fig. 2. The vitamin A activity of carotene under various dietary modifications.

————: Arachis oil diet.
– – – – –: "Fat-free" diet.
(*a*): 1 drop radiostol daily.
(*−a*): Radiostol withdrawn.
(*b*): 1 drop irradiated ergosterol in paraffin (same conc.) daily.

Experimental.

For these experiments two sets of young albino rats were used.

Set I. Of nine rats five (including one as the negative control) received the basal diet containing 15 % of arachis oil which has been generally employed in this research, the remaining four received a similar, but "fat-free," diet differing only in the substitution of additional rice starch in place of arachis oil, and in the substitution of medicinal paraffin for arachis oil as a solvent for

the irradiated ergosterol in those cases in which an addition of vitamin D was made. When growth had failed carotene (prepared from carrots; M.P. 174°) was administered at the level of 0·01 mg. daily at the points shown, being dissolved either in arachis oil (by warming) or in medicinal paraffin (by addition of the pigment in chloroform solution and concentration under diminished pressure).

It will be seen from the growth curves (Fig. 2) that the negative control rat declined and died, whereas in all the rats receiving carotene decline was prevented and growth restored. Poor growth responses, however, were given in some cases, and notably in those in which the carotene was administered in medicinal paraffin.

Set II. Of six rats, three received the ordinary basal diet containing arachis oil and vitamin D, three received the "fat-free" diet without the addition of vitamin D. After growth had ceased in both groups 0·01 mg. of carotene dissolved in arachis oil was administered in all cases and simultaneously the diets given were interchanged as indicated in the growth curves. Except in the case of one animal, which died suddenly, growth was restored in both groups.

The results indicate that carotene is active even in the absence of substantial amounts of fat in the basal diet. The definitely positive responses obtained when carotene was administered in paraffin solution demonstrate, moreover, that the process previously adopted of dissolving the pigments in warm arachis oil was not an essential in promoting physiological activity. It is clearly a matter of minor importance, in this connection, that activity tended to be weakened by the use of paraffin, since in any case it has been stated by various workers that the administration of paraffin leads to a less ready utilisation of vitamin A.

SUMMARY.

1. Carotene (M.P. 162°) obtained from red palm oil was active as a source of vitamin A activity to rats in doses of 0·01 mg. daily.

2. Carotene (M.P. 174°) obtained from carrots was inactive as a source of vitamin D to rats in doses greatly in excess of the minimal dose for vitamin A.

3. Carotene (M.P. 174°) at the level of 0·01 mg. daily displayed activity even when a basal diet of the "fat-free" type was used. The pigment still displayed activity when medicinal paraffin replaced arachis oil as a solvent for the test doses, although this modification tended to weaken the growth responses observed.

My thanks are due to Dr L. J. Harris for his most helpful criticism.

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CXLI. REGULARITIES IN THE GLYCERIDE STRUCTURE OF VEGETABLE SEED-FATS.

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(*Received November 6th, 1929.*)

THERE is now in existence a large amount of evidence that simple triglycerides, containing a single fatty acid, are only formed in any quantity in a natural fat if no other method of combining the fatty acids at the disposal of the plant or animal is possible.

Qualitative work on the fractional crystallisation of fats by Klimont [1902, 1903, 1904, 1905, 1912], Bömer [1907, 1909, 1913, 1920] and Amberger [1913, 1918, 1923, 1924], in the cases of cacao butter, beef and mutton tallows, butter, lard and other fats, and particularly Bömer's fractional crystallisations of coconut and palm kernel fats [Bömer and Schneider, 1924], proving the absence of trilaurin from both of them, although their lauric acid content is nearly 50 % of the total mixed acids, all show the wide extent of this generalisation.

Another body of evidence is provided by the work of Suzuki and Yokoyama [1927, 1928], and of Eibner [1927, 1928], on linseed, soya-bean, cod-liver and whale oils. By fractional crystallisation of the bromine-addition products of the glycerides present in these oils they have isolated, from linseed oil, dilinoleolinolenin, oleodilinolenin, and two varieties of linoleodilinolenin; from soya-bean oil, oleodilinolenin, linoleodilinolenin, dilinoleolinolenin, oleolinoleolinolenin and dipalmito-olein; and they have shown that similar mixtures occur in cod-liver and whale oils. The most interesting feature, however, is the almost complete absence of simple triglycerides, which brings these oils into harmony with the more saturated fats examined by the former group of workers.

During the past few years considerable attention has been given in our laboratory to the more saturated fats and it has been found possible quantitatively to remove any unsaturated glycerides by means of oxidation of the fat with anhydrous potassium permanganate in acetone solution, a procedure which converts all but the fully saturated triglycerides present into acidic products, and thus leads to a quantitative determination of the amount of completely saturated triglycerides in the original fat. Furthermore, when the compositions of the mixed fatty acids in the fully saturated glycerides and in the original fat have been ascertained, it becomes possible to obtain a measure of the average number of molecules of saturated fatty acid which are associated with one molecule of unsaturated acid in the mixed saturated-unsaturated

glycerides (plus tri-unsaturated glycerides) of the original fat; and, again, from this may be deduced limiting ranges for the proportions of mono-unsaturated-disaturated, di-unsaturated-monosaturated, and tri-unsaturated glycerides present in the original fat.

By taking advantage in this way of the amenability of unsaturated fatty acid residues to chemical change (oxidation), we are accumulating a good deal of evidence as to general relationships in the distribution of fatty acids in combination with glycerol, from the point of view of the manner in which saturated and unsaturated acids are associated. There is little, if any, reason to suppose that these relationships will differ fundamentally from those which govern the partition of individual saturated or unsaturated acids amongst the glycerol residues of a natural fat; on the contrary, the general similarity of all the higher fatty acids, saturated or unsaturated, in so far as matters of esterification or hydrolysis are concerned, justifies to some extent[1] the inference that relationships between saturated and unsaturated acids will largely hold for all the individual members of either group. At present, however, so far as semi-quantitative experimental investigation goes, we are confined almost exclusively by the chemical properties of the fats to observation of the two types of glyceride (i) fully saturated, and (ii) mixed saturated-unsaturated or wholly unsaturated.

The results which have been obtained by this method have verified, and have also brought out a difference in degree of, the tendency towards even distribution of fatty acids throughout a fat, which was indicated by the earlier work referred to at the commencement of this paper. We find that there is a

[1] So far as can be judged at present, it seems almost certain that the distribution of fatty acids in mixed saturated glycerides is governed by the same rules which operate in the saturated-unsaturated acid partition in mixed saturated-unsaturated glycerides; so that in seed-fats there is notably even distribution (cf. the examination of the fully saturated glycerides of coconut or palm kernel fat [Collin and Hilditch, 1928], in which trilaurin is not present, although lauric acid forms 50–60 % of the fatty acids), whilst in animal fats there is a more heterogeneous kind of assemblage in the fully saturated as in the whole of the glycerides (thus mutton tallow, the fatty acids of the fully saturated portion of which contain 50 % palmitic and 44 % stearic acid, contains 3 % of tristearin and possibly some tripalmitin [Collin, Hilditch and Lea, 1929], a result confirmed by Bömer [1907]; and tripalmitin or tristearin has been similarly reported by Bömer and by Amberger in certain other animal fats).

Concurrently with these general features, however, there is evidence that specific fatty acids may associate predominantly together in different ways in different fats. In some cases the composition of the saturated fatty acids (i) associated together, and (ii) associated with unsaturated acids in mixed saturated-unsaturated glycerides is much the same (*e.g.* dika fat in this paper, p. 1281), whilst in others it is essentially different. Thus in mutton tallow and cacao butter palmitic acid tends to concentrate in the fully saturated glycerides (as also in the case of *Myristica malabarica* fat, this paper, p. 1285), whilst in other cases (cf. nutmeg butter and laurel oil, this paper, pp. 1283–1287) it has been found mainly associated with the unsaturated acids.

This specific character of the association of different fatty acids in varying types of fat, which may be compared with the known specificity of fatty acid composition in the fats of certain botanical orders of plants [Hilditch, 1928], is apparently not in conflict with the operation of the general laws which govern the assemblage of the mixed fatty acids into triglycerides according to the biological origin of the fat, animal or vegetable.

marked difference between animal and vegetable fats, particularly vegetable seed (kernel) fats, in that the tendency referred to is much stronger in vegetable than in animal fats.

This is most clearly to be appreciated from a consideration of the relative number of molecules of saturated fatty acids present per molecule of unsaturated fatty acid in the whole original fat and in the portion thereof which does not consist of fully saturated triglycerides. To avoid repetition of a necessarily lengthy phrase, we shall refer to the number of molecules of saturated fatty acids present per molecule of unsaturated acid (more especially when in association in the form of mixed saturated-unsaturated triglycerides) as the "association ratio." The numerical value of the "association ratio" appears, from such data as are yet available, to be quite distinctly characteristic of the biological origin of the particular fat.

Up to the present, we have obtained information from less than twenty fats of widely varying type, but more detailed work is in progress on non-seed fats of vegetable origin (such as palm oil) and on a considerable number of animal fats of different kinds. We have, however, already completed a more thorough examination of a range of solid vegetable seed-fats, the results of which form the subject of this communication; it is in this group that the "association ratio" reaches the highest values observed so far, each molecule of oleic acid being linked, circumstances permitting, with an average of 1·3–1·6 molecules of saturated acid. In other words, the mixed saturated-unsaturated glycerides consist largely of mono-oleo-disaturated glycerides with some di-oleo-monosaturated glycerides and the even distribution of the fatty acids amongst the glycerol residues appears to reach a maximum in this class of fats.

The figures on which we base these deductions are summarised in Table I, wherein are also included, for the sake of illustration, such data as are yet available for vegetable non-seed fats and for various kinds of animal fats. Column I gives the fat examined, column II the percentages by weight of saturated and unsaturated fatty acids in the original fat, and column III the percentage of fully saturated triglycerides in the original fat, whilst the "association ratios" of the (molecular) proportions of saturated to unsaturated acids in the whole fat and in the mixed saturated-unsaturated (plus tri-unsaturated) glycerides are given respectively in columns IV and V.

In connection with Table I, we would point out that the association ratios for the mixed glycerides of kusum, cottonseed and ground-nut oils and of lard and rabbit fat have not been worked out in so much detail as in the other cases, owing to the small proportions of fully-saturated material present; whilst all the figures for beef tallow must be regarded as of a preliminary nature. Any uncertainties thus present in the values in column V for these six fats (which are consequently placed within brackets) are, however, sufficiently small not to interfere with their presentation in contrast to the remaining seed-fats.

Table I.

Fat examined	Acids in whole fat: Sat. %	Acids in whole fat: Unsat. %	Fully saturated triglycerides %	"Association ratios": In whole fat	"Association ratios": In sat.-unsat. glycerides	Reference
I	II		III	IV	V	VI
Vegetable seed-fats:						
Coconut	91·7	8·3	84	15·5	1·3–1·4	Collin and Hilditch [1928]
Dika	89·4	10·6	79	11·0	1·3	See this paper, p. 1280
Myristica officinalis	88·6	11·8	71	9·2	1·6	See this paper, p. 1281
Palm kernel	80·8	19·2	63	5·8	1·3–1·4	Collin and Hilditch [1928]
Illipé tallow	62·0	38·0	4·5	1·7	1·55	Hilditch and Priestman (forthcoming publication)
Cacao butter	58·8	41·2	2·5	1·5	1·4	Lea [1929]
Myristica malabarica	54·9	45·1	18	1·5	1·0	See this paper, p. 1283
Kusum oil	35·5	64·5	1–2	0·6	(0·6)	Dhingra, Hilditch and Vickery [1929]
Cottonseed oil	23·4	76·6	1 (?)	0·33	(0·33)	Hilditch and Lea [1927]
Ground-nut oil	15·1	84·9	1 (?)	0·18	(0·18)	Christian and Hilditch (forthcoming publication)
Vegetable non-seed fats:						
Palm oils	46·9	53·1	9	0·97	0·8	Hilditch and Jones (not yet published)
	48·6	51·4	10	1·04	0·8	
	41·6	58·4	7	0·78	0·65	
Laurel oil	44·7	55·3	26	1·09	0·37	See this paper, p. 1285
Animal fats:						
Butter fats	61·3	38·7	31	2·06	1·05	Hilditch and Jones [1929]
	59·9	40·1	29	1·95	1·04	
Mutton tallow	59·7	40·3	26	1·6	0·9	Collin, Hilditch and Lea [1929]
Beef tallow	51·0	49·0	14	1·1	(0·8)	Christian and Hilditch (not yet published)
Lard	44·1	55·9	8	0·85	(0·7)	Hilditch and Sleightholme (not yet published)
Rabbit fat	31·9	68·1	7	0·5	(0·4)	Vickery (private communication)

The main distinction which we wish to draw between animal and vegetable (seed) fats is sufficiently clear when cacao butter and Illipé tallow are compared with the two butter fats and mutton tallow. The unsaturated acid content of all five fats is almost equal, but, whereas the animal fats contain saturated glycerides varying between 26–31 %, the corresponding figures for the two vegetable fats are 2·5–4·5 %. It also appears that a similar distinction may be drawn between vegetable fats which are derived from seeds or kernels and those originating from other parts of the plant. Palm oil, a fruit pulp fat, and laurel oil (probably from a similar source but possibly mixed with a little seed-fat) show distinct differences in their glyceride construction from the other vegetable fats of seeds or kernels.

The clearest idea of the differences in the distribution of the fatty acids (saturated and unsaturated) is given by the association ratios in column V, which distinguish sharply between the animal fats and non-seed vegetable fats and the ten different varieties of seed-fats which have now been studied.

The data for the animal fats (the three last members of which have as yet only received preliminary investigation) show that in these there is markedly

less evenness of distribution of the fatty acids than in either class of vegetable fats; and such results as we have obtained up to the present indicate that uneven partition of the fatty acids with glycerol may be equally characteristic of vegetable fats originating elsewhere than in the seed itself. Laurel oil, with which (although not a seed-fat) we deal in this paper, appears to stand out quite exceptionally in this respect.

Turning now to the group of ten vegetable seed-fats, the "association ratio" of the saturated to unsaturated acids in the whole fats is equal to or greater than 1·5 : 1 in seven instances (three of which represent additional fats selected for study of their glyceride structure from the standpoint of the present communication); and in all but one of these the corresponding association ratio in the non-fully saturated glycerides present lies between 1·3 and 1·6 : 1, although the association ratio for the acids of the whole fat ranges from 1·5 to 15·5 : 1.

The remaining three fats contain preponderating amounts of oleic acid, and it is impossible at this juncture to discriminate between the amount of this acid present as triolein and that associated with saturated acids in mixed glycerides. The important point to observe in these liquid fats is the almost entire absence of fully saturated glycerides in spite of the presence of appreciable quantities of saturated acids (15–35 % by weight). The phenomenon of "even distribution" is evidenced here, as it were, from the opposite side: so long as there is sufficient excess of oleic acid present (in kusum oil the molecular ratio of oleic to saturated acids is about 1·8 : 1), all saturated acids are absorbed into mixed saturated-unsaturated glycerides.

It is not unreasonable to infer from this that, in those fats where the total molecular ratio of saturated : unsaturated acids exceeds the latter figure (say 2 : 1), triolein is similarly almost completely absent, so that from palm kernel fat upwards (in the table) the substantial absence of triolein may be presumed. The association ratio in the mixed glycerides is nevertheless of the same order as in the cases of Illipé tallow and cacao butter, thus again pointing to the general evenness of distribution of the fatty acids throughout kernel fats.

The principle of even distribution is seen at its maximum in cacao butter and Illipé tallow, where the proportions of saturated and unsaturated acids in the whole fats only slightly exceed the favoured mixed-glyceride "association ratio" of 1·3–1·6 : 1 and wherein, in point of fact, practically the whole of the fats are made up of mono-oleo-disaturated and di-oleo-monosaturated glycerides. Full details of the investigation of Illipé tallow will be published shortly elsewhere, but it may be said here that both this fat and cacao butter are further characterised by the presence of oleic, palmitic and stearic acids in proportions not far removed from the same molecular order, and that study of their oxidation products by special methods (of which a description has been given by Lea [1929] when dealing with cacao butter) has shown definitely that very large proportions of oleopalmitostearins, with subsidiary amounts of oleodistearins and oleodipalmitins, are present.

The fat from the seeds of *Myristica malabarica* is the only one of its type so far examined which does not fall within the scope of the generalisation suggested above. It is closely related, from the botanical standpoint, to nutmeg butter (the association ratio of which is in keeping with the rest) and the sample examined is undoubtedly a genuine kernel fat (prepared by extraction in this laboratory from nuts of authentic origin). The molecular ratio of saturated to unsaturated acids in the whole fat is much the same as in cacao butter, but the proportion of fully saturated glycerides is much higher (18 %) and the association ratio for the remainder of the fat is only 1 : 1. For the moment this must be counted as an exceptional case; it may be observed that this particular oil was accompanied by unusually large amounts of resinous and other non-fatty matter and that the mottled appearance of the kernels is some indication of unusual heterogeneity in the contents of the endosperm cells.

A few words may be added with reference to the specimen of laurel oil, the examination of which (although it is not a seed-fat) has been included in this paper owing to the interesting structure which has been disclosed. The mixed fatty acids (35 % lauric, 10 % palmitic, 36 % oleic, 19 % linoleic—molecular ratio of saturated : unsaturated, 1·1 : 1) were in proportions which would permit the association of all the saturated acid with the unsaturated acids in mixed glycerides, yet oxidation showed the presence of 26 % of fully saturated triglycerides. From the composition of the total fatty acids it follows, of course, that if saturated glycerides are present at all they must consist mainly of trilaurin; it is not the presence of trilaurin but rather the high proportion of fully saturated glycerides which is surprising, with its concurrent implication that the unsaturated acids are likewise linked with each other in an unusual degree. The low "association ratio" of 0·37 : 1 for the non-fully saturated glycerides connotes, in fact, the presence of between 15 and 48 % of triunsaturated glycerides, according to the respective amounts of di-oleo-monosaturated and mono-oleo-disaturated glycerides which may also occur in the fat.

Whether this predisposition to extreme heterogeneity is peculiar to the Lauraceae or is a frequent occurrence in vegetable non-seed fats cannot be known until this class of fats has received much more than the scanty attention so far given to them; but it may be pointed out as a matter of general interest that laurel oil is the only example we have met of a fat whose structure at all resembles the former erroneous conception of natural fats as a mixture of relatively simple triglycerides.

We would draw attention, in conclusion, to the two fats, laurel oil and nutmeg butter, wherein (from our estimations of the fully saturated glyceride contents and the composition of the saturated acids present in these fragments of the whole fats) we find evidence of the presence respectively of nearly 25 % of trilaurin and about 50 % of trimyristin. It is interesting to note that from these fats, which by chance contain large amounts of simple triglycerides,

Bömer and Ebach [1928] were able to isolate, by the fractional crystallisation process, quantities of these components of much the same order, namely, 30 % of trilaurin from laurel oil and 40 % of trimyristin from nutmeg butter; on the contrary, in spite of prolonged and laborious quantitative crystallisations, Bömer and the other workers failed to isolate more than insignificant quantities of any simple triglyceride from fats such as coconut, palm kernel, butter, tallow, etc.—all cases in which our own methods and the generalisations to which they have led us indicate that substantially only mixed glycerides are present. Bömer's procedure and our own, although fundamentally different in character, thus lead satisfactorily to exactly the same general conclusions.

Experimental.

The experimental methods employed in studying the four additional fats for which data are now given in this paper—dika fat, nutmeg butter, *Myristica malabarica* fat and laurel oil—were the same as those which have been given at length in several communications on glyceride structure from these laboratories (for example, in that [Collin and Hilditch, 1928] dealing with the component glycerides of coconut and palm kernel fats). It is thus unnecessary to recapitulate these in detail and the experimental figures given below are confined for the most part to a brief summary of the final results obtained.

So far as the determination of the composition of mixed fatty acids is concerned, we give only the final summary of the proportions estimated to be present in the "solid" and "liquid" acids (as obtained after separation by the lead salt-alcohol process) from the observed results of fractional distillation of the neutral methyl esters of each group. Fuller details of these analyses will be given elsewhere later for certain of these fats, the composition of which is of some general technical interest.

As regards the permanganate-acetone oxidations carried out in order to estimate the proportion of fully saturated glycerides present, the final weight of the latter is given after making the necessary corrections for any small amounts of acidic products of oxidation left in the purified neutral compounds. The only points of difference in the procedure now used from that adopted for coconut and palm kernel fats [Collin and Hilditch, 1928] were:

(i) the use of aqueous sodium carbonate for the separation of the oxidised fat into neutral and acidic portions was superseded by that of ammonia, which was found to have less tendency to produce emulsification;

(ii) in most of the present instances, the amount of fully saturated glycerides available was too small to permit of final purification by boiling with aqueous alkali carbonate solution, and in consequence traces of mono-azelaic glycerides were probably present in the neutral compounds when analysed for fatty acid composition. Correspondingly, traces of dimethyl azelate would be present in some of the methyl esters fractionated, leading to slightly lower saponification equivalents for the first fractions; thus the lowest fraction of

these esters from the fully saturated glycerides of laurel oil had a mean equivalent of 208·5, whereas the analysis of the original mixed fatty acids showed that no esters lower than methyl laurate (equivalent 214·0) were present. In such instances the fractions in question (in all cases small) were taken as being substantially methyl laurate.

1. Dika fat.

Dika fat is the kernel-fat from various species of *Irvingia*. The sample investigated was prepared by extracting with ether the kernels of a small consignment of the fruit of *I. Barteri*, which the Director of Forests, Ibadan, Nigeria, had been good enough to collect for us. Dika fat has previously been examined by Lewkowitsch [1905], who considered it to be a mixture of laurin and myristin with a very little olein.

The characteristics of the specimen we studied, and Lewkowitsch's corresponding data, are as follows:

	Present sample	Lewkowitsch
Saponification equivalent ...	233·9	224–232
Iodine value	9·07 %	4·3–5·2 %
Unsaponifiable matter	1·05 %	—
M.P.	41–42°	38·9–41°
Mixed fatty acids (free from unsap.)		
Mean equivalent	220·3	—
M.P.	37·5–38°	—

Less than 70 g. of extracted fat was available for the complete investigation, and hence the fractionation and oxidation processes were perforce carried out on a much smaller scale than usual; the relative simplicity of the fatty acid mixture present and the high proportion of fully saturated glycerides, on the other hand, made it unnecessary to work with very large quantities of material in this particular case.

Composition of the total fatty acids of dika fat.

In view of the small quantity available and the very small proportion of unsaturated acids, the usual lead salt separation was omitted. The methyl esters of the mixed acids were resolved into six fractions by distillation under a high vacuum.

Lauric acid (anilide, M.P. 73·5°) was identified in the lowest fraction; the melting-point of the acid itself could not be raised above 36·5° by repeated crystallisation, although admixture with authentic lauric acid (M.P. 43°) raised the melting-point to 37–38°. We favour the view that traces of some lower acid rendered purification of the lauric acid unusually difficult, rather than the very unlikely alternative that an isomeric form of lauric acid is present in *Irvingia* fat.

The unsaturated acid present in small quantity was not definitely identified by conversion into a dihydroxystearic acid, but there is no reason to consider that it is other than the normal oleic acid.

The estimated composition of the total fatty acids was lauric (38·8 %), myristic (50·6 %) and oleic (10·6 %).

Oxidation of dika fat.

The fat (40 g.) was dissolved in acetone (400 cc.) and oxidised with finely-powdered potassium permanganate (80 g.), and finally yielded 31·55 g. (corrected weight) of neutral products (fully saturated glycerides), or 78·8 % of the whole fat.

Since 100 parts of original fat (sap. equiv. 233·9 and unsap. matter 1·05 %) gave 79 parts of fully saturated glycerides (sap. equiv. 228·9 and unsap. matter 0·7 %), it follows that the "association-ratio" in the non-fully saturated glycerides of the fat is 1·34 mols. saturated : 1 mol. oleic acid.

Composition of fatty acids in the fully saturated glycerides.

Methylation and fractionation of these fatty acids led to their composition being established as lauric (42·8 %) and myristic (57·2 %) acids. Lauric acid prepared from the two lowest ester fractions again possessed a low melting point, viz. 38·5° after three crystallisations (mixed with pure lauric acid, M.P. 38–41°); myristic acid was obtained from the fifth fraction (M.P. 52·5°, and, mixed with authentic myristic acid, 53°).

Distribution of the fatty acids in dika fat (estimated from the fractionation analyses).

	Total fat 100 g.	Fully sat. glycerides 79 g.	Mixed sat.-unsat. glycerides (by difference) 21 g.
Unsaponifiable matter	1·1	0·6	0·5
Glyceryl residue	5·4	4·3	1·1
Lauric acid	36·2	31·7	4·5
Myristic acid	47·4	42·4	5·0
Oleic acid	9·9	—	9·9

The data in the final column correspond with an "association ratio" of 1·3 : 1.

Percentage compositions of the saturated fatty acids present.

	(i) In the whole fat	(ii) In fully sat. glycerides	(iii) In the mixed glycerides
Lauric acid	43·3	42·8	47·5
Myristic acid	56·7	57·2	52·5

2. NUTMEG BUTTER (*Myristica officinalis*) FAT.

The fat examined was a specimen of guaranteed genuine origin supplied by Messrs Evans Sons, Lescher and Webb, Ltd. It contained 17·7 % of unsaponifiable matter and possessed an iodine value of 61·0 % (mainly due to essential oil), whilst the mean equivalent of the crude fatty acids present was 246·3.

Power and Salway [1907, 1908] gave the composition of nutmeg fat as essential oil (mainly *d*-pinene and *d*-camphene, 12·5 %), trimyristin (73·0 %),

triolein (3·0 %), trilinolein (0·5 %), resin (2·0 %) and unsaponifiable matter (8·5 %), whilst Bömer and Ebach [1928] isolated from it 40 % of trimyristin, M.P. 56·2°.

For our purposes we removed as much volatile oil as possible from the original pasty, brown fat by distillation with steam; the residue (about 70 % of the crude fat) was boiled with excess of aqueous sodium carbonate, well washed with boiling water and recovered, when it was much harder, and paler in colour, than the original fat, but still possessed an iodine value of 46 and saponification equivalent 295. These values were found to be mainly due to unsaponifiable matter which passed into solution in alcohol during the subsequent lead salt separation, and which was largely removed, prior to fractionation of the liquid acids as methyl esters, by converting the liquid acids into sodium soaps and extracting the aqueous solution of the latter repeatedly with ether.

Composition of the mixed fatty acids of nutmeg butter.

The following figures were thus finally obtained:

	"Solid" acids 71·0 %	"Liquid" acids 29·0 %	Total	Fatty acids % composition
Lauric acid	1·0	0·2	1·2	1·5
Myristic acid	60·1	1·5	61·6	76·6
Palmitic acid	8·2	—	8·2	10·1
Oleic acid	1·7	6·7	8·4	10·5
Linoleic acid	—	1·1	1·1	1·3
Unsaponifiable	—	19·5	19·5	—

From the main fraction of esters obtained on refractionation of the second primary fraction, myristic acid (M.P. 52·5–53·5°) was obtained after one crystallisation (mixed with authentic myristic acid, M.P. 52–53°), whilst the main fractions of acids from esters of the "liquid" acids yielded by oxidation with cold alkaline permanganate the characteristic dihydroxystearic acid corresponding with oleic acid, M.P. 128–129°.

Oxidation of nutmeg butter.

The refined fat (100 g.) was dissolved in acetone (1000 cc.) and treated in the usual manner with powdered potassium permanganate (400 g.); a large excess of permanganate was used in the hope that the non-fatty matter still present would be converted by oxidation into alkali-soluble products. This, as a matter of fact, took place to a large extent, since the final product contained only 1 % of non-fatty matter, as compared with 18·7 % in the material oxidised.

100 g. of the refined fat (containing 81·3 g. glycerides of mean saponification equivalent 248·1) gave 58·6 g. (corrected weight) of neutral products (fully saturated glycerides containing 1 % of non-fatty matter). Calculated on the glycerides originally present, the proportion of fully saturated components is therefore 71 %; the "association ratio" in the non-fully saturated glycerides, calculated from these figures, is 1·60 mol. saturated : 1 mol. oleic acid.

Composition of fatty acids in the fully saturated glycerides.

By fractionation of the methyl esters (35·6 g.) of the acids from the purified fully saturated material, it was estimated that there were present lauric (2·3 %), myristic (90·9 %) and palmitic (6·8 %) acids.

Distribution of the fatty acids in nutmeg butter (estimated from the fractionation analyses).

	Total fat 100 g.	Fully sat. glycerides 58·6 g.	Mixed sat.-unsat. glycerides (by difference) 41·4 g.
Unsaponifiable matter	18·7	0·6	18·1
Glyceryl residue ...	4·2	3·1	1·1
Lauric acid	1·2	1·2	—
Myristic acid... ...	59·0	50·0	9·0
Palmitic acid ...	7·8	3·7	4·1
Oleic acid	8·1	—	8·1
Linoleic acid... ...	1·0	—	1·0

The data in the final column correspond with an "association ratio" of 1·7 : 1. (The mean value 1·65 : 1 for the nutmeg butter "association ratio" corresponds with a mixture of 87 mols. mono-oleo-disaturated and 13 mols. di-oleo-monosaturated glycerides, if no triolein is present.)

Percentage composition of the saturated fatty acids present.

	(i) In the whole fat	(ii) In fully sat. glycerides	(iii) In the mixed glycerides
Lauric acid	1·7	2·2	—
Myristic acid ...	86·8	91·1	68·7
Palmitic acid ...	11·5	6·7	31·3

Owing to the very small proportion of mixed glycerides present in the fat, the values in the last column (which depend on small differences between relatively large numbers) are probably less reliable than the remainder.

3. *Myristica malabarica* fat.

The kernels of *M. malabarica* contain a fat in which oleic acid occurs in much higher proportion than in that from the related *M. officinalis*, the respective "association ratios" for the total fatty acids being 1·5 mol. and 9·2 mols. of saturated acids per mol. of unsaturated acids. The fat examined was prepared by extraction with carbon tetrachloride from seeds supplied by the District Forest Officer, Malabar District, Madras Presidency; it was a very dark-coloured, almost liquid fat and contained large amounts of resinous and non-fatty matter, which were extracted, as far as possible, by washing the fat (in ethereal solution) with aqueous ammonia and subsequently boiling it with excess of concentrated sodium carbonate solution. The fat, after refining in this manner, was semi-solid at the ordinary temperature, but still dark in colour; its apparent saponification equivalent (unsaponifiable matter still present) was 280, and its iodine value 77 %.

Composition of the mixed fatty acids of Myristica malabarica *fat.*

The following figures were obtained by analyses conducted on similar lines to those used for the nutmeg butter acids:

	"Solid" acids 36·2 %	"Liquid" acids 63·8 %	Total	Fatty acids % composition
Myristic acid ...	18·2	4·1	22·3	39·2
Palmitic acid ...	6·0	1·6	7·6	13·3
Other saturated acids	1·3	—	1·3	2·4
Oleic acid	2·7	22·4	25·1	44·1
Linoleic acid ...	—	0·6	0·6	1·0
Non-fatty matter ...	8·0	35·1	43·1	—

Palmitic acid (M.P. 60°) was identified in the penultimate fraction of distilled methyl esters of the "solid" acids, whilst from the residue from this distillation there was obtained in small amount the dihydroxystearic acid (M.P. 128°) corresponding with oleic acid and a mixture of saturated acids which, after three recrystallisations, melted alone at 63°, but, when mixed with pure palmitic acid, at 54–55°; for the purposes of calculation, the higher acid present was taken as stearic acid. Oleic acid was recognised by oxidising the acids from several of the "liquid" ester fractions with cold alkaline permanganate, when dihydroxystearic acid, M.P. 130°, was readily obtained.

The residue from the fractional distillation of the "liquid" esters contained most of the non-fatty matter originally present and was, of course, very large, in spite of carrying the distillation as far as possible by intensive heating; it was submitted to oxidation both by the cold alkaline permanganate process and by anhydrous permanganate in boiling acetone solution, and no evidence of the presence of more than traces of retained methyl oleate was obtained in either case. Consequently it was reckoned entirely as non-fatty matter in the calculation.

Oxidation of Myristica malabarica *fat.*

The oxidation by means of potassium permanganate in acetone solution was not completed in one operation and it was therefore necessary to submit the primary neutral products to a repetition of the process. Finally, from the refined fat (264·35 g.) there was obtained 28·3 g. (corrected weight) of fully saturated glycerides, or 10·7 % (without correction for unsaponifiable matter).

Thus, 100 parts of original material (containing 58·1 parts of glycerides, mean equivalent 255·0) yielded 10·7 parts of fully saturated material (containing 3 % non-fatty matter, mean equivalent of fatty compounds present 243·3); the "association ratio" in the non-fully saturated glycerides is thus 0·99 mol. saturated : 1 mol. unsaturated acids.

Composition of fatty acids in the fully saturated glycerides.

This was determined, by fractionation analysis of the methyl esters (15 g.), to be myristic (43·9 %), palmitic (54·4 %) and stearic (1·7 %) acids.

The main component of the earlier fractions of esters was methyl myristate (myristic acid, M.P. 52°, unchanged when mixed with authentic myristic acid,

after two crystallisations), whilst the residue consisted mainly of methyl palmitate with a small proportion of the methyl ester of a higher acid (calculated as stearic acid).

Distribution of the fatty acids in Myristica malabarica *fat (estimated from the fractionation analyses).*

	Total fat 100 g.	Fully sat. glycerides 10·7 g.	Mixed sat.-unsat. glycerides (by difference) 89·3 g.
Unsaponifiable matter	41·9	0·3	41·6
Glyceryl residue ...	2·7	0·5	2·2
Myristic acid... ...	21·7	4·3	17·4
Palmitic acid ...	7·4	5·4	2·0
Stearic acid	1·3	0·2	1·1
Oleic acid	24·4	—	24·4
Linoleic acid... ...	0·6	—	0·6

The data in the final column again correspond with an "association ratio" of 0·99 : 1.

Percentage composition of the saturated fatty acids present.

	(i) In the whole fat	(ii) In fully sat. glycerides	(iii) In the mixed glycerides
Myristic acid... ...	71·4	43·4	84·9
Palmitic acid ...	24·3	54·6	9·7
Stearic acid	4·3	2·0	5·4

4. Laurel fat.

The specimen investigated was supplied by Messrs Evans Sons, Lescher and Webb, Ltd. and had the following characteristics:

Saponification equivalent	269·8
Acid value	9·0
Iodine value	86·4 %
Unsaponifiable matter	6·2 %
Mean equivalent of mixed fatty acids (freed from unsaponifiable matter)	249·5

In the earlier literature [cf. Matthes and Sander, 1908] this fat was considered to be composed almost entirely of trilaurin and its high iodine value to be due to the presence of essential oil; whilst the latter doubtless contributes to the observed iodine value, we now show that over half of the total fatty acids are made up of oleic and linoleic acids [cf. also Lewkowitsch-Warburton, 1922].

Bömer and Ebach [1928] isolated 30 % of trilaurin, M.P. 45·6°, from laurel oil by fractional crystallisation of the fat from ether and acetone.

The oil which we examined was semi-solid and was dark green in colour; it was refined, prior to investigation, by boiling with aqueous sodium carbonate and subsequent thorough washing with boiling water.

Composition of the mixed fatty acids of laurel fat.

The following figures were obtained:

	"Solid" acids 50·7 %	"Liquid" acids 49·3 %	Total	Fatty acids % composition
Lauric acid	23·5	8·2	31·7	35·0
Palmitic acid ...	8·8	—	8·8	9·7
Oleic acid	12·9	20·1	33·0	36·6
Linoleic acid	2·5	14·4	16·9	18·7
Unsaponifiable matter	3·0	6·6	9·6	—

The fractionation of the "solid" methyl esters was abnormal in that between the second fraction (28·3 g., B.P. 87°/1 mm., sap. equiv. 214·9) and the fourth (17·9 g., B.P. 125–127°/1 mm., sap. equiv. 274·3) there was only a very small intermediate fraction (7·0 g., B.P. 87–125°/1 mm., sap. equiv. 253·8) covering a very wide range of boiling-point. Special attention was paid to the fatty acids present in this ester-fraction: palmitic acid (M.P. 62·5°) was readily obtained in quantity, but the more soluble portions present on further crystallisation eventually gave palmitic acid (M.P. 61–62°), except for one crop from the original mother liquors, which yielded a small amount of acid, M.P. 53–55°. This, however, on admixture with myristic acid, melted indefinitely at 44–46·5°. We conclude, therefore, that myristic acid is either absent, or present only in very small quantities; and we have based the analytical calculations on a mixture of lauric and palmitic acids.

Lauric acid (M.P. 43–44°) was isolated readily from the lower ester-fractions, and palmitic acid (M.P. 62–62·5°) from several of the higher fractions of the "solid" esters.

The main fractions of the liquid esters were hydrolysed and oxidised with ice-cold aqueous alkaline permanganate solution, when a tetrahydroxystearic acid soluble in hot water (M.P. 149–150°), a tetrahydroxystearic acid insoluble in hot water or ethyl acetate (M.P. 169–171°) and a dihydroxystearic acid soluble in hot ethyl acetate (M.P. 128–129°) were obtained in each case examined. These are the characteristic products of alkaline oxidation, under the conditions described, of ordinary oleic and linoleic acids.

Oxidation of laurel fat.

The refined fat (100 g.) was oxidised as in the cases previously described and the primary neutral products submitted to re-oxidation; there were finally produced 26·4 g. (corrected weight) of fully saturated glycerides. Allowing for unsaponifiable matter present in the material oxidised, this leads to an "association ratio" in the non-fully saturated glycerides of 0·37 mol. saturated per mol. unsaturated acids.

Composition of fatty acids in the fully saturated glycerides.

This was determined to be: lauric (94·0 %) and palmitic (6·0 %) acids.

Distribution of the fatty acids in laurel fat (estimated from the fractionation analyses).

	Total fat 100 g.	Fully sat. glycerides 26·4 g.	Mixed sat.-unsat. glycerides (by difference) 73·6 g.
Unsaponifiable matter	9·2	0·3	8·9
Glyceryl residue ...	4·5	1·6	2·9
Lauric acid	30·2	23·0	7·2
Palmitic acid ...	8·4	1·5	6·9
Oleic acid	31·6	—	31·6
Linoleic acid	16·1	—	16·1

The "association ratio" for the non-fully saturated glycerides from these data is the same as that given above, 0·37 : 1.

It should be observed that this figure is consonant with the presence of 81 mols. of di-unsaturated-monosaturated and 19 mols. of tri-unsaturated glycerides, or with that of 40 mols. of mono-unsaturated-disaturated and 60 mols. of tri-unsaturated glycerides in the non-fully saturated portion. This implies that in the whole fat there must be (by weight) at least 15 % of tri-unsaturated glycerides and there may be as much as 48 %; whilst the amounts of mono-unsaturated-disaturated and di-unsaturated-monosaturated glycerides must correspondingly lie between nil and, respectively, 26 % and 59 %.

Percentage composition of the saturated fatty acids present.

	(i) In the whole fat	(ii) In fully sat. glycerides	(iii) In the mixed glycerides
Lauric acid	78·2	94·0	50·7
Palmitic acid	21·8	6·0	49·3

Summary.

1. Sufficient natural fats have now been examined with reference to their glyceride structure to justify the general statement that animal fats are built up on more heterogeneous lines than vegetable seed-fats, whilst it is beginning to appear that vegetable fats from parts of the plant other than the seed itself resemble the animal fats rather than kernel fats so far as the distribution of fatty acids amongst the glycerides is concerned.

2. Previous study of four seed-fats (coconut and palm kernel fats, cacao butter and Illipé tallow), in which the total molecular ratio of saturated to unsaturated acids is equal to or greater than 1·5 to 1, has disclosed the presence therein of fully saturated glycerides in amounts varying from 2·5 % to 84 % as the association ratio referred to increases from 1·5–15·5 to 1, and, correspondingly, the occurrence of saturated and unsaturated acids in mixed glycerides in proportions denoted by molecular association ratios varying from 1·3–1·55 to 1—an almost constant ratio in comparison with the widely differing characteristics of the fats in other respects.

In the present paper three further fats of similar total association ratio for the saturated and unsaturated acids have been investigated, and two of these

have been found to conform closely with the foregoing rules, whilst the third (*Myristica malabarica* fat) was found to be somewhat exceptional.

3. Three other seed-fats (kusum, cottonseed and ground-nut oils) which contain preponderating amounts of unsaturated acids, and have already been studied from this point of view, are all characterised by the almost complete absence of any fully saturated glycerides, although the proportion of saturated fatty acids in the whole fat ranges from 15 to 35 % in the oils in question.

4. All these data illustrate the pronounced tendency to even distribution of the fatty acids throughout the glycerides of seed-fats as a class. If the molecular proportion of unsaturated to saturated acids in the whole fat exceeds about 1·6 : 1, the saturated acids are almost wholly present in the form of mixed glycerides; if the aforesaid molecular proportions are reversed, increasing amounts of fully saturated glycerides are found to be present, but in every case but one so far examined the mixed saturated-unsaturated glycerides present contain saturated and unsaturated acids in molecular association ratios lying within the comparatively narrow limits of 1·3–1·6 to 1 (usually 1·3–1·4 to 1); finally, when fully saturated glycerides are present in quantity, no simple triglyceride has been detected (even when one acid forms 30–50 % of the whole of the saturated acids, *e.g.* lauric acid in coconut and palm kernel fats or palmitic and stearic acid in cacao butter or Illipé tallow) unless the composition of the saturated fatty acids is so simple that one acid is present in overwhelming excess (*e.g.* myristic acid in nutmeg butter, which contains a considerable proportion of trimyristin).

5. Laurel fat, although not a true seed-fat, has been included in this communication because of its interesting glyceride structure, which is strongly heterogeneous: although the molecular proportions of saturated and unsaturated acids in the whole fat are approximately equal, the fat contains 26 % of fully saturated glycerides (largely trilaurin) and the molecular association ratio for the acids of the mixed glycerides is 0·37 to 1, connoting the presence of tri-unsaturated glycerides in quantity.

6. Consideration of all these vegetable fats by the experimental methods described leads to the conclusion that simple saturated triglycerides exist in only two of them, namely, nutmeg butter and laurel fat; and it is of great interest to note that exhaustive fractional crystallisation of a number of these solid fats in the hands of Klimont, Bömer and their co-workers has given negative results in the isolation of simple saturated triglycerides, except in the two cases mentioned. In these instances, however, Bömer and Ebach [1928] isolated trimyristin and trilaurin respectively in amounts which are of the same quantitative order as the proportions disclosed by our present study.

We wish to express our thanks to the Department of Scientific and Industrial Research for a grant made to one of us (G. C.) throughout the course of this work.

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CXLII. THE CHEMICAL ASPECT OF THE DESTRUCTION OF OAK WOOD BY POWDER POST AND DEATH WATCH BEETLES—*LYCTUS* SPP. AND *XESTOBIUM* SP.

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(*Received November 6th, 1929.*)

Introduction.

In considering the extensively destructive activities of the larvae of wood-boring beetles, and granting that during boring operations wood is pulverised and passed through the alimentary canals of the organisms, the important question arises as to whether wood substance, in part or in whole, is utilised as food. In the study of wood-boring organisms such as white ants, it has been proved by comparing analyses of sound wood and borings respectively that in virtue of the symbiotic activity of intestinal micro-organisms the larvae concerned actually digest a portion of the carbohydrate components of the wood in which they bore [Schorger, 1926, pp. 503–4; Hawley and Wise, 1926, pp. 302–3]. These conclusions have been arrived at by comparing the carbohydrate-lignin ratio in sound wood and borings respectively. In the case of the white ants this ratio was found to be lower in the borings than in sound wood, hence the deduction that carbohydrate material was removed. Moreover, by assuming that lignin was not attacked, and remained constant throughout, it was possible to calculate the actual percentage of such material digested.

With regard to the feeding habits of wood-boring insects in general, there is some doubt from the entomological standpoint as to whether they invariably feed on wood substance. Indeed Uvarov [1928] has summarised the position as follows: "There is, however, no evidence at all as to what substances they do in fact feed on; these may be particles of wood partially digested by micro-organisms, or their secretions, or their excretions, or even the micro-organisms themselves. In other words wood-eating insects must be classified as insects of absolutely unknown feeding habits."

In the present investigation an attempt has been made to determine the chemical effect, if any, (1) of the larva of a species of powder post beetle on oak sapwood, and (2) of the larva of the death watch beetle on oak heartwood.

Experimental.

Series I. The material used was *Lyctus* frass or borings collected from a number of samples of infected English oak sapwood. For the purposes of comparison a sample of sound sapwood was obtained from a disc of English oak cut at about 20 ft. from the ground.

Comparative analyses of frass and sound wood were carried out according to the methods recommended by Schorger [1926, pp. 505–6] and Hawley and Wise [1926, pp. 129–72], the percentages of the main constituents only being determined. In screening the frass, prior to analysis, it was found that the material was of a texture fine enough to permit of its passage through a 120 mesh screen. Since standard wood analyses are as a rule carried out on 80–100 mesh material, the composition of the sample of sound English oak sapwood was first determined on material of this particle size. It can be seen from the following tables that particle size has a marked influence on analytical results, since in the 120 mesh material the hot water- and alkali-soluble contents respectively are higher, and the cellulose, lignin, and pentosan contents are lower than those in the 80–100 mesh material prepared from the same sample of sound sapwood. These discrepancies are no doubt explained by the fact that in grinding the wood to fine particle size, greatly increased surface area is exposed to analytical reagents. A true comparison between the respective compositions of sound wood and frass, taken from the same species of wood, is thus afforded in Table II.

Results are expressed throughout as percentages by weight of oven-dry material.

Table I. *Analysis of sound English oak sapwood of 80–100 mesh.*

	%
Hot water-soluble	9·9
Alkali-soluble	22·6
Cellulose	55·0
Lignin	22·4
Total pentosans	25·1

Table II. *Comparative analyses of* Lyctus *frass from English oak sapwood and sound English oak sapwood of same particle size.*

Material	Hot water-soluble	Alkali-soluble	Cellulose	Lignin	Total pentosans
Sound English oak sapwood, 120 mesh	10·4	24·2	52·9	21·8	24·3
Lyctus frass, 120 mesh	11·5	23·0	52·8	21·1	25·4

Series II. The material used was *Xestobium* frass collected from a badly infected sample of English oak heartwood. The frass was in the form of small pellets which crumbled very easily to a fine dark brown powder. Light grinding in a mortar was sufficient to reduce the whole sample to a powder which passed a 120 mesh screen. The composition of this material is given in Table III.

For the purpose of comparison a standard wood analysis was carried out on a sample of 80–100 mesh well-seasoned English oak heartwood taken 20 ft. from the ground (Table III).

Table III. *Analysis of* Xestobium *frass from English oak heartwood and of 80–100 mesh English oak heartwood.*

	Xestobium frass	Oak heartwood
Hot water-soluble	13·6	13·0
1 % alkali-soluble	57·3	24·5
Cellulose	39·0	53·2
Lignin	32·1	26·5
Total pentosans	16·5	21·7

Discussion.

Series I. The analytical data show clearly that *Lyctus* frass taken from the sapwood of English oak sapwood is of the same general composition as sound English oak sapwood of the same particle size. The agreement of the data is close considering that the sound wood and frass, although obtained from the same species of wood, were none the less obtained from different trees.

Since the carbohydrate-lignin ratio in *Lyctus* frass is of the same order as that in sound wood, it would appear that the larva does not feed on any particular wood constituent, for in the light of previous work, if any of the main components were interfered with during larval activity the carbohydrate-lignin ratio in the frass would betray the fact. Since the composition of the actual wood from which the frass was obtained could not be determined, it was thus impossible to determine any loss in weight which might have been sustained as a result of attack. There remains the open possibility therefore, that if a loss in weight were sustained, part of the wood substance might have been consumed *in toto*. That lignin could be utilised by the larva as food, however, seems highly unlikely, and since cellulose and pentosans appear on analysis to be in no sense depleted, the conclusion that the *Lyctus* larva does not feed on the wood in which it bores seems justified. Moreover, having regard to the remarks by Uvarov [1928] previously quoted, it would appear that among all the possible sources of food available to the larvae, "micro-organisms themselves or cell contents are most likely to be used." Thus the larvae must feed directly on some cell contents of sapwood or else on some micro-organisms which feed on cell contents, for comparative analysis shows that the main constituents of wood cell-wall substance are not affected by larval activity.

Series II. With regard to the effect of the death watch beetle on the heartwood of oak, the experimental data in Table III show clearly that, in view of the low cellulose and pentosan contents of the frass, part of these components in the original wood must have been consumed during larval activity. The high alkali-solubility of the frass is further evidence that some radical change has taken place in the wood substance during attack.

Since the composition of the original wood, and the loss in weight after attack could not be determined, it is impossible definitely to establish the true

extent of depletion in each component, but comparison of the carbohydrate-lignin ratios is conclusive evidence of attack on the carbohydrates of the original wood.

The experimental procedure adopted in the above studies of the feeding habits of two wood-boring beetles is admittedly of limited application since it can only be used within broad limits to indicate whether part of the skeletal substance of wood can be used as food by the larvae in question. None the less, as a preliminary to further and more detailed study of the biology of wood-boring organisms it is of unquestioned value. For instance, with regard to the powder post beetles the analytical results point to the fact that the source of larval nourishment is within the cells of the sapwood of oak and not in the cell-wall substance.

Treatment of death watch beetle frass by the same technique discloses the fact that the larva in this case attacks the carbohydrates of wood substance. The indication here is therefore that intestinal micro-organisms play a prominent part in the biology of the larva. When it is possible to feed the larvae of wood-boring insects on weighed samples of wood of known composition, so that the losses in weight due to attack can be determined, the full effectiveness of the application of chemical analysis to the elucidation of feeding habits will be realised, since it is obvious that accurate information on this subject must lead to more efficient methods in wood preservation.

I wish to express my indebtedness to Prof. F. Soddy for facilities afforded in the Old Chemistry Department, University of Oxford, to Sir James Irvine and Dr J. W. Munro, for helpful criticisms and advice, and to Mr R. S. Pearson, Director of Research, Forest Products Research Laboratory, Princes Risborough, Bucks., for permission to publish these results.

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CXLIII. THE VITAMIN D PROBLEM[1].

I. THE PHOTOCHEMICAL REACTIONS OF ERGOSTEROL.

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(*Received September 14th, 1929.*)

In the course of the last year several publications have appeared, *e.g.* by Kon, Daniels and Steenbock [1928], Webster and Bourdillon [1928], in which the authors came to the conclusion that the photochemical reaction of ergosterol, caused by irradiation with ultra-violet light, is independent of the wave-length used in the range from $300\mu\mu$ onward. Our own experiments on this subject, which have since been repeated and refined, prove the incorrectness of this conclusion, for we found that irradiation with light of wave-length $> 275\mu\mu$ gives rise to a series of reaction products which differs from that obtained by irradiation with light of wave-length $254\mu\mu$. This follows from the fact that the changes in the absorption spectrum are different in the two cases.

Experimental.

(1) *Absorption spectra.* In order to obtain the absorption-indices, we have chosen the photographic method, as worked out under the direction of Prof. L. S. Ornstein in the Physical Laboratory of Utrecht, for measuring intensities [cf. Dorgelo, 1925]. The photographs were taken with a source of light which emitted the continuous spectrum of hydrogen. It was constructed substantially according to the prescriptions given by Bay and Steiner [1927]. The use of this very suitable source of light enabled us to evaluate the absorption spectra at arbitrary wave-lengths, and thus to locate exactly all singularities of the absorption curves. The quartz spectrograph was one by Hilger, type E 2; the photographs were measured with an especially constructed rapid-recording photoelectric photometer.

(2) *Irradiation.* In our earlier experiments we irradiated the ergosterol, dissolved in hexane, in a glass tray with a cover-plate of quartz. In order to exclude oxygen, argon was continually blown over the solution. As we learned however, from further experiments, that the smallest traces of oxygen might influence the results, we afterwards used only all-quartz *cuvettes*. These

[1] The present paper is the first of a series of three, which present the results of a joint research carried out at the Pharmaco-Therapeutical Institute of the University, Leyden, by Prof. W. Storm van Leeuwen, J. W. R. Everse and J. van Niekerk, and at the Physical Laboratory of the N. V. Philips' Gloeilampenfabrieken, Eindhoven, by ourselves.

were filled with the solution, cooled in solid CO_2, evacuated with a high-vacuum pump, and then sealed off. In these latter experiments the absorption spectra were taken immediately after the irradiation, without opening the *cuvette*, in order to prevent oxidation after irradiation.

As the production of pure monochromatic radiation of sufficient intensity is very difficult, we have provisionally limited our research to experiments with two different ranges of wave-lengths, which may be obtained separately with appropriate light-sources and filters. The following light-sources were used.

(*a*) A quartz-mercury arc (Heraeus).

(*b*) A condensed magnesium spark.

(*c*) A mercury column in argon, which has been worked out in this laboratory: under definite conditions this column emits very strongly the resonance-line $253{\cdot}7\,\mu\mu$, whilst the other lines are weak.

The above light-sources were used in combination with the following filters.

I. A quartz-tray of thickness 0·5 cm., filled with a 10 % solution of benzene. This filter cuts off all radiation of wave-length $< 275\,\mu\mu$, whereas the other radiation is scarcely weakened.

II. A quartz-tray, of thickness 2 cm., filled with saturated chlorine vapour of 15° (pressure ca. 6 atm.). This filter, whilst transmitting the line $254\,\mu\mu$ for about 90 %, absorbs the longer wave-lengths very strongly, so that between $\lambda = 275\,\mu\mu$ and $\lambda = 390\,\mu\mu$ less than 0·1 % is transmitted.

III. A quartz-tray, of thickness 0·5 cm., filled with a 0·5 % solution of KNO_3, in distilled water. This filter absorbs all radiations shorter than $241\,\mu\mu$, whereas the other radiation is scarcely weakened.

Two separate ranges of wave-lengths were obtained.

(1) $\lambda > 275\,\mu\mu$, by combining the mercury arc (*a*) or magnesium spark (*b*) with the benzene filter (I).

(2) $\lambda = 254\,\mu\mu$, by combining the mercury column (*c*) with the chlorine filter (II) and the KNO_3 filter (III).

The ergosterol was prepared from yeast and purified *via* the acetate. Its melting-point was 163° in air and 165° *in vacuo*. As already mentioned, hexane was used as a solvent because of its small absorption (the absorption beginning only beyond $200\,\mu\mu$); moreover it may be considered as a highly indifferent solvent, as has been proved in many cases by V. Henri and his collaborators. During the irradiation, the solution was vigorously stirred by an iron stirrer, which was operated by means of an electromagnet.

Experimental results.

The changes in the absorption spectrum of a solution irradiated with light of $\lambda > 275\,\mu\mu$ ("long-wave" irradiation) are quite different from those caused by irradiation with light of $\lambda = 253{\cdot}7\,\mu\mu$ ("short-wave" irradiation). This difference becomes more and more marked as the duration of the irradiation

becomes longer, but is already clearly visible after a short exposure to ultraviolet light. In order to interpret the photochemical changes by means of the resulting absorption spectra, it would be desirable to know the absorption-curves of all the substances formed. This constitutes the main difficulty, as it appears that the reaction does not stop after the formation of a first product, but that this product itself is subsequently changed, and it is not possible to separate all these products.

In order to find the absorption curve of the first-formed substance, we have chosen the following way. The absorption-curve was measured of a solution irradiated for such a short time that most of the ergosterol was left unchanged. Afterwards the amount of unchanged ergosterol was evaluated by precipitation with digitonin. On the assumption that the irradiation has not lasted long enough to cause a noticeable conversion of the first reaction product, the absorption curve of this substance can be determined as the difference between the absorption curves of the irradiated solution and of the unchanged fraction of ergosterol. As long as the curves deduced in this way from solutions irradiated during increasing lengths of time prove to be identical (of course after reduction to the same concentration), it follows that the product has not changed.

This method, of course, does not answer the question whether the first reaction product is a single substance or a mixture of substances, formed simultaneously. This would be the case, *e.g.*, if the ergosterol molecule, after absorbing a quantum of radiant energy, had different possibilities of rearrangement. This method moreover does not give insight into the further photochemical changes, only an indication of their existence.

No objection can be made against the above procedure, as the absorption spectra are taken in the absence of the damaging influence of oxygen, and as the estimation of the unchanged ergosterol may be considered to be unimpaired by oxidation of the reaction product.

The most obvious way to determine the absorption spectrum of the reaction product, *i.e.* measuring the absorption of the solution after precipitation of the unchanged ergosterol, is a very risky one. Indeed we found it only practicable, if every precaution had been taken to exclude even traces of oxygen during the whole process. The best proof that the precautions have been successful lies in the fact that the absorption spectrum established in this way tallies with that determined in the previously described way.

(*a*) *Long-wave irradiation.* Fig. 1 gives the absorption curve[1] of an unirradiated ergosterol solution (concentration 80 mg./l.), Figs. 2–10 those of solutions irradiated during 1, 3, 7, 15, 45 minutes, $1\frac{3}{4}$, 13, 25 and 40 hours respectively. These figures show clearly that during the first 15 minutes the

[1] The absorption coefficient a is given by: $a = -1/d \log \text{nat } I/I_0$, where I_0 and I represent the intensities of incident and transmitted light (after correction for losses by reflection on the walls of the *cuvette* and for the absorption of the solvent); d measures the thickness of the layer in mm.

irradiation substantially causes an increase in absorption over the whole range; while later on the absorption diminishes in the long-wave range, whereas it remains about the same at shorter wave-lengths. One very interesting fact however is, that the absorption in the maximum at 282 $\mu\mu$ diminishes from the beginning, while in that at 271·5 $\mu\mu$ the first change is an increase in absorption. This proves conclusively that for the first reaction product the absorption index at 282 $\mu\mu$ is smaller than that for ergosterol, whereas at 271·5 $\mu\mu$ it is larger. Fig. 11 gives the absorption curve of this substance, which we will provisionally call L, calculated as described above, for the concentration of 80 mg./l. As a consequence of a possible accumulation of errors associated with this calculation the curve obtained may be incorrect in minor details, but it certainly is substantially correct. The dots and the crosses are calculated from different irradiated solutions, and agree very well. Further, it can be shown in the following way that as long as the degree of conversion of the solution does not exceed a certain amount the first reaction product is not destroyed.

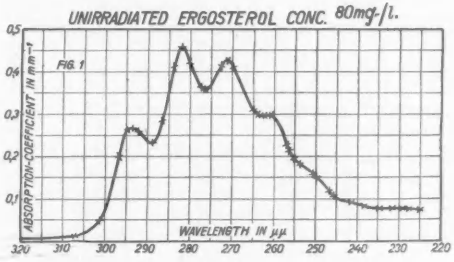
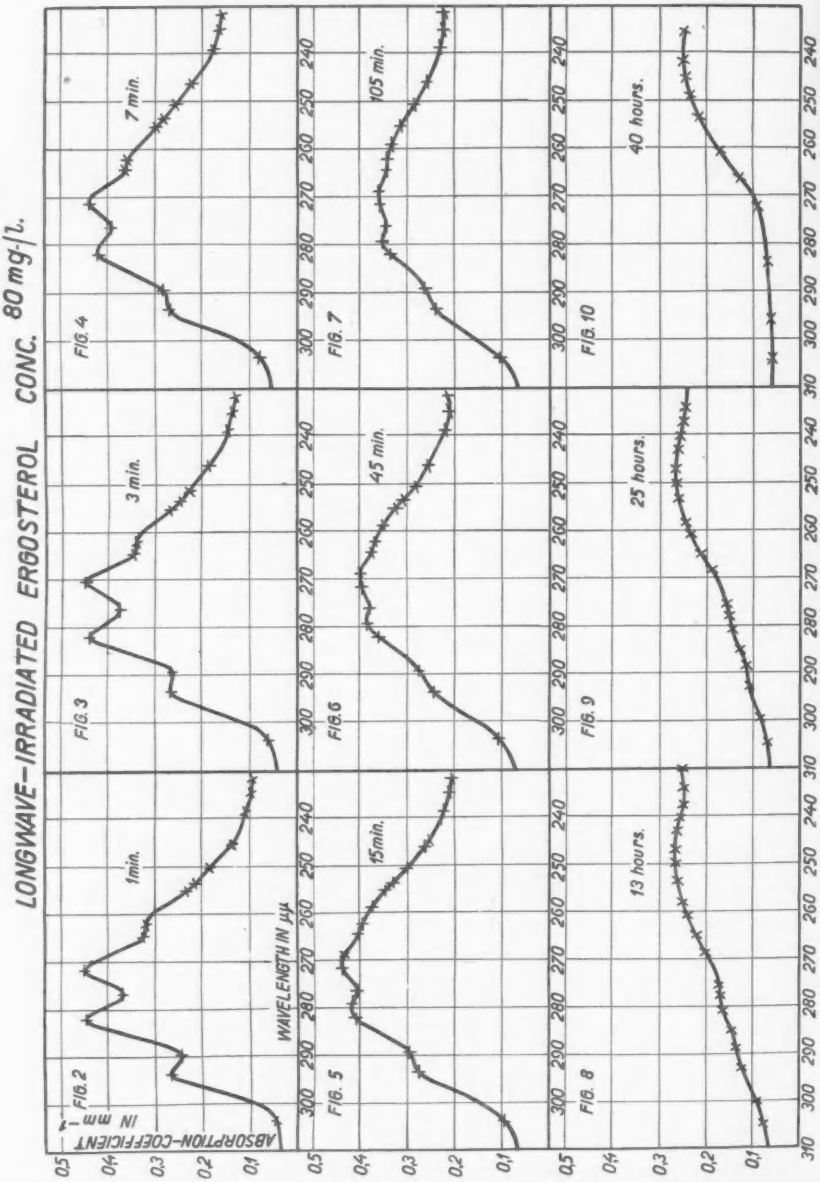


Fig. 1.

Since for an arbitrary wave-length the absorption indices are now known for ergosterol and for the substance L, say α_1 and α_2, the absorption index of an irradiated solution, say α_i, must satisfy the condition: $\alpha_i = x\alpha_1 + y\alpha_2$ in which x and y represent the relative amounts of unchanged and changed ergosterol respectively. This is true as long as no secondary reaction takes place. As analogous equations hold for all wave-lengths (with other values of α but with the same x and y), we can write down a system of equations that must be satisfied simultaneously by the same values for x and y. By applying this procedure to the absorption curves Figs. 2, 3, 4 and 5, and solving for x and y by the method of the least squares, we found the figures given in Table I.

Table I. *Calculated composition of ergosterol exposed to long-wave irradiation.*

Time of irradiation mins.	Ergosterol %	Substance L (vitamin D) %	Sum %
1	90·0	10·4	100·4
3	77·1	22·8	99·9
7	64·8	34·7	99·5
15	48·8	50·5	99·3


LONGWAVE-IRRADIATED ERGOSTEROL CONC. 80 mg-/l.
ABSORPTION-COEFFICIENT IN mm⁻¹
WAVELENGTH IN μμ
FIG. 2
1 min.
FIG. 3
3 min.
FIG. 4
7 min.
FIG. 5
15 min.
FIG. 6
45 min.
FIG. 7
105 min.
FIG. 8
13 hours.
FIG. 9
25 hours.
FIG. 10
40 hours.

Figs. 2–10.

With the aid of these values for x and y, the absorption curves of the irradiated solutions can be calculated. Table II shows the very close agreement between the calculated and experimental absorption indices.

Table II. *Comparison of calculated and experimental absorption coefficients* (a), *of ergosterol exposed to long-wave irradiation.*

Wave-length ($\mu\mu$)	Irradiation 1 min.			Irradiation 3 mins.		
	a exp.	a calc.	Difference in %	a exp.	a calc.	Difference in %
234·8	0·098	0·100	−2	0·135	0·132	+2
238·8	0·110	0·111	−1	0·146	0·146	0
246·1	0·141	0·143	−1·5	0·184	0·182	+1
250·7	0·187	0·187	0	0·224	0·224	0
253·7	0·215	0·211	+3	0·247	0·248	−0·5
255·2	0·231	0·224	+2	0·265	0·260	+2
262·3	0·319	0·322	−1	0·340	0·345	−1·5
264·3	0·320	0·326	−2	0·341	0·350	−2·5
271·5	0·449	0·437	+3	0·447	0·444	+1
276·3	0·365	0·367	−1	0·371	0·378	−2
282·0	0·446	0·452	−1·5	0·439	0·442	−1
289·3	0·241	0·242	−0·5	0·260	0·250	+4
293·5	0·267	0·265	+1	0·265	0·261	+1·5
	Irradiation 7 mins.			Irradiation 15 mins.		
234·8	0·165	0·163	+1·5	0·209	0·204	+2·5
238·8	0·177	0·179	−1	0·221	0·224	−1·5
246·1	0·221	0·220	+0·5	0·266	0·270	−1·5
250·7	0·257	0·261	−1·5	0·305	0·309	−1
253·7	0·280	0·282	−1	0·329	0·331	−1
255·2	0·295	0·294	+0·5	0·348	0·340	+2
262·3	0·359	0·369	−3	0·395	0·401	−1·5
264·3	0·360	0·372	−3·5	0·406	0·405	0
271·5	0·435	0·448	−3	0·438	0·459	−5
276·3	0·392	0·387	+1·5	0·408	0·403	+1
282·0	0·418	0·430	−3	0·412	0·420	−2
289·3	0·281	0·258	+8	0·291	0·269	+8
293·5	0·271	0·258	+4·5	0·273	0·254	+7

The fact that in Table I the sum of x and y is very nearly equal to 1, is a further proof for the correctness of our assumptions. If this method of calculation is applied to the curves of Figs. 6–10, the results are no longer satisfactory. This means that the conditions on which the calculations are based do not hold in this case, *i.e.* part of the first reaction product itself has been destroyed. In agreement herewith, the figures show that prolongation of the irradiation after at least 50 % of the ergosterol has been converted causes a gradual diminution of the absorption in the range about 270 $\mu\mu$. This change, however, is much slower than the one first mentioned, and indicates a relative stability of the substance L toward this long-wave radiation. After 25 hours of irradiation only small traces of bands at about 290 $\mu\mu$ and 280 $\mu\mu$ are visible. These disappear completely after 40 hours, whilst the resulting absorption curve then shows an ill-defined band between 250 $\mu\mu$ and 240 $\mu\mu$. In experiments in which the oxygen has not been carefully excluded, the absorption in this range proves to be much more marked.

After this stage has been reached, the photochemical reaction slows down still further, subsequent changes, if present, being hardly noticeable.

In the case of long-wave irradiation we succeeded in preparing the first reaction product, free from ergosterol, in such a way that its absorption spectrum was identical with that calculated above (see Fig. 11, where the unbroken line represents the measured absorption curve of this substance).

This was done by performing all operations in a metal box in which the air was replaced by a mixture of nitrogen and hydrogen, carefully freed from oxygen by leading it over platinised asbestos at 250°.

This product will be shown later to be vitamin D. It can be obtained in a crystalline state, but the melting point of the crystals is well below 0°. At room temperature the substance has the appearance of a colourless vitreous mass.

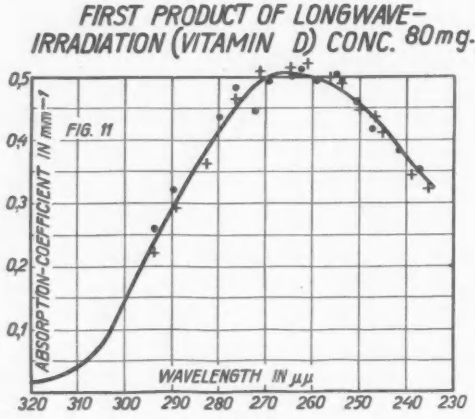


Fig. 11.

As the low melting-point makes further investigation of the crystals rather difficult, we restrict ourselves at the moment to the statement that the crystals have the appearance of lozenge-shaped plates.

(*b*) *Short-wave irradiation.* That short-wave irradiation gives rise to a series of reactions different from those caused by long-wave irradiation, follows from a comparison of Figs. 2–10 with Figs. 12–17, in which the absorption-spectra of a solution of 80 mg./l., irradiated during $2\frac{3}{4}$, 7, 14, 60,135 and 330 minutes respectively, with short-wave light are shown. (The exposures in the two series are not directly comparable, as the intensities of the light-sources are different.)

Now, after $2\frac{3}{4}$ minutes' irradiation, the absorption has increased over the whole range of wave-lengths, 280 $\mu\mu$ included. Prolongation of the irradiation causes this increase to be much more marked, until after 14 minutes the absorption at longer wave-lengths is much higher than in any case of long-wave irradiation. The most striking features of the change are the gradual disappearance of the band at 262 $\mu\mu$, and the large increase of the bands at 282 $\mu\mu$ and 293 $\mu\mu$ with respect to that at 271·5 $\mu\mu$.

Here, too, prolonged exposure to radiation causes a further photochemical change, in this case betraying itself by a gradual decrease of the absorption in the whole range of wave-lengths which was explored. After 330 minutes no appreciable absorption is left.

In the case of long-wave irradiation we obtained an insight into the reaction mechanism by initially calculating the absorption spectrum of the first reaction product in the way outlined on p. 1296. This method appears to be impracticable here, as the calculated absorption spectrum is not independent of the degree of conversion of the solution. This shows that secondary reactions take place even when only little of the ergosterol has been changed.

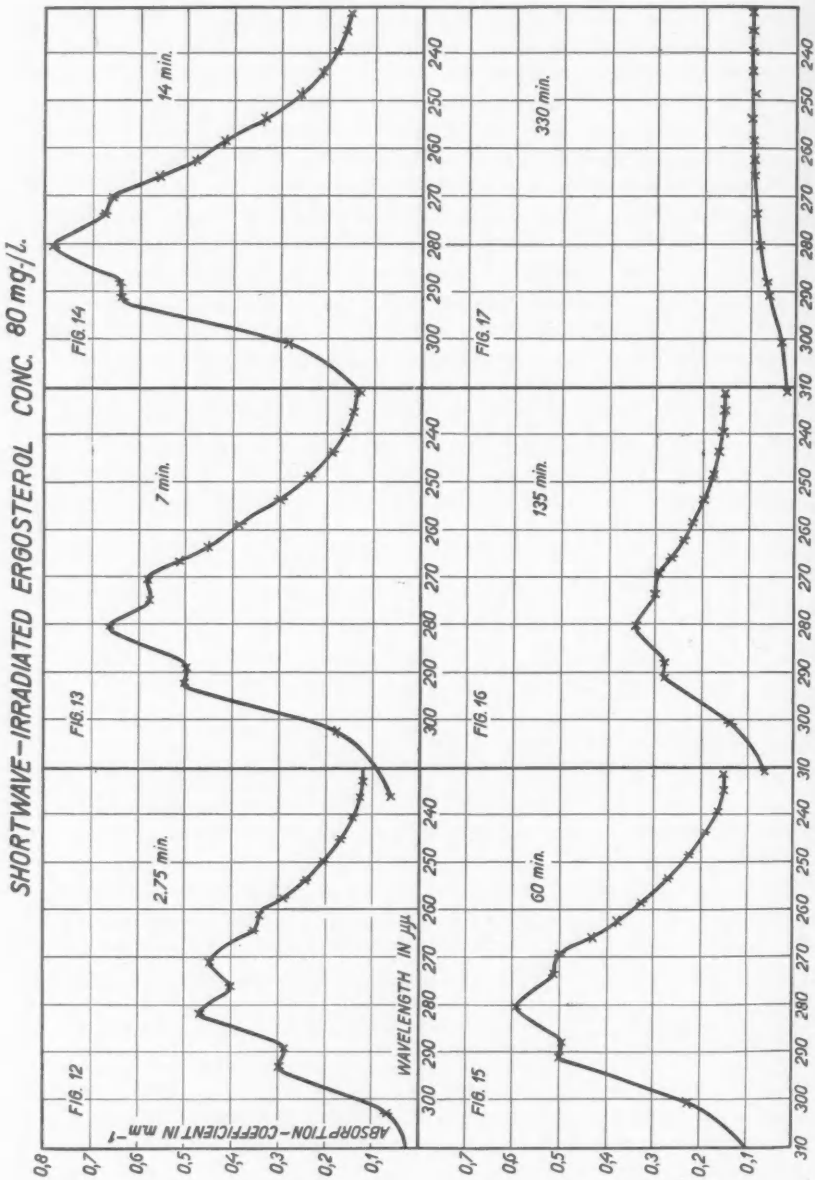
However, there appears to be another way to attack the problem. This is shown by a quantitative comparison of the curves of Figs. 14, 15 and 16. Table III shows that in the wave-length range $> 262\,\mu\mu$, the ratios of the absorption indices of these three curves are independent of the wave-length. From this we conclude that, when the reaction has come to the stage characterised by Fig. 14, the absorption in this range of wave-length is due to one substance only, which upon prolonged irradiation is gradually transformed into a substance without appreciable absorption in this range. This is in agreement with Fig. 17, which shows the absorption spectrum of nearly the final stage.

Table III. *Ratio of the absorption coefficients* (a) *after* 60 *and* 135 *minutes of short-wave irradiation, to those after* 14 *minutes of irradiation.*

Wave-length ($\mu\mu$)	a 60/a 14	a 135/a 14
262·2	0·79	0·50
265·7	0·78	0·47
270·0	0·77	0·45
273·6	0·75	0·43
280·3	0·75	0·43
288·0	0·77	0·43
291·1	0·78	0·43
300·7	0·81	0·48
311·1	0·75	0·50

The curve of Fig. 14 may thus be considered as the absorption spectrum of a single product, formed in the course of the reaction. For the sake of convenience we denote this product by the letter S. We do not know the precise concentration of the solution for which Fig. 14 holds. However, as it follows from the foregoing, that this substance S is destroyed by the radiation, evidently this concentration is less than 80 mg./l. (*i.e.* the concentration of the original ergosterol solution).

Now we know already that in the earlier stages of the reaction there must be at least three absorbing substances present. Ergosterol and the substance S are very likely to be two of them. As we have no knowledge of the relative amounts in which they are present, we cannot, in order to evaluate the spectrum of a third substance, use a method similar to that described before. Yet we did not think it unreasonable to try whether the absorption curves


SHORTWAVE-IRRADIATED ERGOSTEROL CONC. 80 mg-/l.
FIG. 12
2.75 min.
FIG. 13
7 min.
FIG. 14
14 min.
FIG. 15
60 min.
FIG. 16
135 min.
FIG. 17
330 min.
ABSORPTION-COEFFICIENT IN m.m⁻¹
WAVELENGTH IN μμ

Figs. 12–17.

could be calculated on the assumption that, besides ergosterol and S, the substance L (see p. 1297) was also present.

On this hypothesis we calculated the relative amounts of these three substances, by the method of least squares. Table IV shows the observed absorption indices and those calculated on this basis for the spectra of Figs. 12 and 13.

Table IV. *Comparison of calculated and experimental absorption coefficients (α) of ergosterol exposed to short-wave irradiation.*

Irradiation 2¾ mins.				Irradiation 7 mins.			
Wave-length ($\mu\mu$)	α exp.	α calc. (65·8 % erg. 13·3 % S 15·6 % L)	Difference in %	Wave-length	α exp.	α calc. (37·6 % erg. 61·4 % S 4·9 % L)	Difference in %
240·5	0·139	0·144	−3·5	239·4	0·160	0·159	+0·5
245·1	0·166	0·162	+2·5	243·7	0·186	0·185	+0·5
249·6	0·203	0·204	−0·5	248·6	0·236	0·231	+2
253·7	0·238	0·239	−0·5	253·7	0·298	0·294	+1·5
257·5	0·288	0·292	−1·5	258·7	0·384	0·386	−0·5
261·0	0·340	0·338	+0·5	263·6	0·450	0·448	+0·5
264·5	0·348	0·347	+0·5	266·6	0·511	0·512	−0
271·0	0·445	0·447	−0·5	270·3	0·579	0·585	−1
276·0	0·400	0·398	+0·5	274·7	0·573	0·580	−1·5
281·5	0·465	0·467	−0·5	280·4	0·661	0·662	−0
289·1	0·284	0·284	0	289·0	0·494	0·493	+0
292·9	0·296	0·295	+0·5	292·0	0·501	0·497	+1

The very close agreement between calculated and observed curves supports our assumption. In order to elucidate the first stage of the reaction, we calculated in the same way spectra obtained after very short periods of irradiation. In Table V a comparison is given between calculated and observed values for a time of irradiation of 45 seconds only. Here too the agreement is very good.

Table V. *Comparison of calculated and experimental absorption coefficients (α) of ergosterol, irradiated during 45 seconds with short-wave light.*

Wave-length ($\mu\mu$)	α exp.	α calc. (90·4 % erg. 0·9 % S 6·1 % L)	Difference in %
238·0	0·089	0·096	−7
242·1	0·110	0·110	0
246·2	0·134	0·129	+4
250·0	0·170	0·168	+1
253·7	0·200	0·198	+1
257·5	0·248	0·257	−3·5
261·1	0·307	0·306	+0·5
264·3	0·314	0·307	+2
271·4	0·424	0·423	+0
276·7	0·355	0·356	−0
282·1	0·445	0·447	−0·5
289·4	0·234	0·233	+0·5
293·7	0·259	0·262	−1

From the amounts of the different substances determined, it follows that in the first period of irradiation mainly the substance L is formed, and only relatively little of S; further, that the rate of destruction of L is also much

greater than that of S, so that after 7 minutes of irradiation the amount of L has already passed its maximum and fallen to a value less than that after 45 seconds of irradiation. The concentration of L never reaches a high percentage in the course of the irradiation.

Without for the moment going into details, we may state that the substance S is readily changed by the action of long-wave light, just as is L by short-wave light. This we learned from special experiments; in a subsequent paper we will return to this subject more in detail.

Antirachitic activity.

The antirachitic activity of different samples of ergosterol exposed to long- or short-wave irradiation has been tested on rats in the Institute of Prof. Storm van Leeuwen, by his collaborators J. W. R. Everse and J. van Niekerk, who will publish the outcome of this research in detail. In the meantime, he has kindly allowed us to communicate here the preliminary results.

For the samples exposed to long-wave irradiation, it was found that in the first period of irradiation the activity increased rapidly, fairly parallel with the amount of the substance L formed, as calculated from the absorption-spectra. After continued exposure, when the substance L is being gradually destroyed, the activity also decreases.

This proves that the substance L is identical with vitamin D.

The minimal daily dose able to cure a rat of rickets in 14 days, was 0·00001 mg. for the most potent sample that was tested. Of this last preparation about 50 % consisted of vitamin D[1].

All samples of ergosterol exposed to short-wave irradiation have an activity of 0·1 or less of the best sample of ergosterol exposed to long-wave irradiation. This activity is just of the order of magnitude we should expect on the basis of the content of substance L (vitamin D) found after short-wave irradiation.

A preparation of ergosterol exposed to long-wave irradiation, containing about 30 % of vitamin D, has been used by Prof. E. Gorter and Dr. J. J. Soer in the Children's Hospital of Leiden (Director Prof. E. Gorter), to cure rachitic children. They kindly permit us to mention here that complete healing was attained in all cases within 14 days by a daily dose of 0·04 mg. This dose is a hundred times smaller than that used in general.

[1] In judging the value of the figures given, account should be taken of the fact that our collaborators Everse and Van Niekerk in their physiological standardisation of our preparations consider as a "curative" dose $\frac{1}{2}$ dose of vitamin D which is able to cure *completely* in 2 weeks rickets induced in young rats by a deficient diet given during the preceding 4 weeks. This detail is of interest since many investigators apply a less severe test and are consequently apt to find relatively lower curative doses.

Discussion.

The original conception, based upon the work of Pohl [1926], that vitamin D is characterised by an absorption band at 247 $\mu\mu$, which was the starting point for the considerations of Morton, Heilbron and Kamm [1927, 1, 2], has of late been abandoned.

The results obtained by Rosenheim and Webster [1927], Webster and Bourdillon [1928], Bills, Honeywell and Cox [1928], Smakula [1928], Windaus, Westphal, Werder and Rygh [1929], Delaplace and Rebière [1929], Lahousse and Gonnard [1929], Fabre and Simonnet [1929] give a high degree of probability to the hypothesis that the irradiation of ergosterol produces several substances in succession, and that the antirachitic activity is connected with the first stage of the reaction. It was especially emphasised by Webster and Bourdillon [1929], that the consecutive changes in the absorption spectrum of ergosterol after increasing periods of irradiation are in agreement herewith. These authors found that an increase in antirachitic activity goes parallel with an increase in the absorption in the range of longer wave-lengths. As has been shown above, the first stage of the photochemical reaction is always accompanied by an increase in the absorption in this range, but while this increase is only comparatively small in the case of long-wave irradiation, it is very marked when short-wave ultra-violet light is used. Now we believe that in the experiments of Webster and Bourdillon [1928] the increase in absorption was mainly due to the action of the short-wave light of the mercury-arc (which light they never absolutely excluded in their experiments on absorption spectra), while the antirachitic activity will have been chiefly caused by the long-wave radiation. Thus we are convinced that the parallelism found by them is mainly accidental. A special experiment showed us that indeed in the earlier stages of the reaction irradiation with the unfiltered light of the mercury arc causes a change in the absorption-spectrum mainly due to the specific action of the short-wave light, *i.e.* the formation of substance *S*.

It is not surprising that the curve which Webster and Bourdillon give for the absorption of the reaction-product, and which they think to be of the type of that of vitamin D, has not much resemblance to our vitamin curve.

The absorption curves given by Windaus *et al.* [1929] have more similarity with our vitamin curve. They all have their maximum at greater wave-lengths however; from this and from their shape it is to be concluded that the products studied by Windaus contained an appreciable percentage of the substance *S*. So, even in the cases where the magnesium spark was used for the irradiation, the short-wave light has been of influence. As a matter of fact, Windaus *et. al.* used the unfiltered light of the spark, in which, though the lines at 280–290 $\mu\mu$ are by far the strongest, those of shorter wave-lengths are not to be neglected.

Kon, Daniels and Steenbock [1928] found that the efficiency of the radiation for the formation of vitamin D from ergosterol was independent of the

wave-length in the range from 256 to 293 $\mu\mu$. Though at first sight there seems to be a discrepancy with our results, a closer inspection of their experimental method shows us, that, on the contrary, their results may be used to support our assumption concerning the influence of the short-wave irradiation. As a matter of fact they irradiated relatively large amounts of ergosterol with monochromatic light for such short durations that only very little of the substance was converted. We conclude for example from their data that in no case more than about 1 % had been changed. On the basis of our calculations it is probable that at such a low degree of conversion practically only vitamin D is formed, as well by short-wave as by long-wave irradiation.

SUMMARY.

1. It is shown that irradiation of ergosterol with light of wave-length 254 $\mu\mu$ ("short-wave" irradiation) gives rise to a series of reaction-products, different from that caused by irradiation with light of wave-length > 275 $\mu\mu$ ("long-wave" irradiation).

2. In the case of long-wave irradiation, the reaction proves to be comparatively simple, in so far as only one product is directly formed from the ergosterol. This product itself, which appears to be vitamin D, is after longer irradiation gradually transformed into a new and much more stable product. As the rate of destruction of the first product is rather small, it is possible to convert about 60 % of the ergosterol into vitamin D before the secondary reaction becomes important.

3. The short-wave irradiation causes changes of a more complex character. In this case there are two reaction-products present during the first period of irradiation.

It is made probable that one of these products, which is very quickly destroyed on prolonged irradiation, and thus does not accumulate to an appreciable amount, is identical with vitamin D. The other product, into which the main portion of the ergosterol is transformed, is much more stable in respect to this irradiation.

4. The absorption spectra of both vitamin D and the "short-wave" product have been evaluated.

5. By means of the absorption spectrum it is possible to estimate with much precision the amount of vitamin D present in correctly irradiated solutions.

6. Feeding-tests on children and rats have shown the first reaction-product, produced by long-wave irradiation, characterised by a distinct absorption spectrum and obtained in crystalline state, to have an antirachitic activity of a magnitude not hitherto reached.

In conclusion we wish to express our thanks to Mr J. Houtgast and Mr H. A. Kruuk for their valuable assistance in the performance of the experiments.

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CXLIV. STUDIES ON COLLAGEN. THE CHANGES WHICH COLLAGEN UNDERGOES WHEN TREATED WITH SOLUTIONS OF HYDROCHLORIC ACID AND SODIUM HYDROXIDE[1].

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(*Received September 24th, 1929.*)

It is a well-known fact that, if a protein is treated with an acid or an alkali, the physical and chemical properties are changed, the degree and nature of these changes depending on the acid or alkali used and on the hydrogen ion concentration.

The first workers in the field, Hardy [1905], Pauli [1922], and Laqueur and Sackur [1902], who investigated the globulins, albumins, and caseinogen respectively, considered that proteins with either an acid or an alkali formed ionisable salts, and attributed the observed increases in certain physical constants to the greater association between the ionised salt and water.

A considerable amount of work has been carried out on other proteins, especially gelatin, by numerous investigators, and from their results it may be concluded that at p_H 2–3, p_H 11–12, and at the isoelectric point there is either a maximum or minimum value for the physical constants, viz., osmotic pressure, viscosity, swelling, specific rotation, etc., and the theoretical aspect of the question has been discussed by Robertson [1920], Dakin [1910, 1912], Leuchs and Manasse [1907], Leuchs and La Forge [1908] and more recently by Levene, Bass, Steiger and Bencowitz [1927].

This paper is mainly concerned with the changes which occur in the precursors of gelatin, when they are treated with solutions of acid or alkali. The investigation is made more difficult by the fact that collagen is insoluble at ordinary temperatures and consequently cannot be brought into solution without altering its internal structure. Therefore most of the facts which are known relating to collagen have been obtained by a study of the gelatin extracted from it. The only simple physical property which can be determined directly is the swelling with change of hydrogen ion concentration. This has been carried out on hide powder by Porter [1921, 1922], Atkin [1922], Kubelka [1918], Kubelka and Taussig [1926], on goat-skin by Kaye and Jordan-Lloyd [1924, 1, 2], and on fish-skin by Kernot and Knaggs [1929].

[1] Thesis presented at the University of London for the Degree of Ph.D.

Knaggs and Schryver [1924, 2] showed that a gelatin derived from a precursor treated with alkali had on hydrolysis a higher percentage of basic nitrogen than one from an acid-treated precursor.

Further, Knaggs [1923] and Kernot and Knaggs [1928, 2] state that proteins treated with cold acid before hydrolysis give results for the Hausmann numbers which indicate that internal structural changes have taken place in the molecule of the protein which markedly affect the distribution of nitrogen, especially in the diamino-fraction.

The author concluded from the above results that, when a tissue swells in solutions of acid or alkali, the swelling is accompanied by intramolecular changes in the molecule of the protein, especially in acid below p_H 2·2 and in alkali above p_H 10·5.

From the work of Jordan-Lloyd and Mayes [1922] and Atkin and Douglas [1924] on the combining power of gelatin with HCl or NaOH, it is noticed that at hydrogen ion concentrations of greater than p_H 1·7 and less than p_H 10 there is a considerable increase in the amount of acid or base fixed.

It was therefore decided to study the precursors of gelatin in order to throw some light on the physical and chemical changes which take place when they are treated with solutions of HCl and NaOH above 0·03 %, and, in particular, 0·5 % HCl and 0·2 % NaOH as these are the concentrations of acid or alkali used industrially, when purifying animal tissues previous to the extraction of the gelatin.

With this object in view the investigations were carried out on the following lines and, to simplify the problem, the collagen used in all these experiments was purified as far as possible from all inorganic and tissue impurities using the method of Knaggs and Schryver [1924, 1].

(1) The purified collagen was treated with 0·5 % HCl or 0·5 % NaOH for a known length of time. The acid or alkali was then removed and the gelatin extracted.

The Hausmann numbers of the gelatin and its precursor, and certain physical properties of the gelatin, were determined to show how far the treatment of the precursor influenced the physical properties and chemical constitution of the gelatin.

(2) The alkali-treated precursor was digested with trypsin to remove the elastic fibres in order to obtain a comparatively pure collagen, and the Hausmann numbers of the collagen so obtained and the gelatin extracted from it were determined.

(3) The swelling of

(*a*) an acid-treated precursor,
(*b*) an alkali-treated precursor,
(*c*) a precursor free from elastic fibres,

in different concentrations of HCl and NaOH was studied and the Hausmann numbers of the swollen tissue were determined, especially in the region of maximum swelling.

Experimental.

Preparation of the materials.

The femur, Achilles tendon and horn-pith of a freshly killed ox were the starting materials. The femur and horn-pith were decalcified by 3 % HCl, and the acid was removed by washing in running water. They then received treatment with 0·2 % NaOH for a prolonged period to remove, as far as possible, all organic impurities [Knaggs and Schryver, 1924, 1].

In the ossein and horn-pith there still remained traces of calcium phosphate which was removed by Smith's method [1921], which consists in treating the tissue with saturated brine and 0·5 % HCl. After washing it, the last traces of electrolyte were removed by electrolysis for 10 days, using the apparatus described by Knaggs, Manning and Schryver [1923] for the purification of gelatin. The collagen thus obtained was practically free from ash.

Method of analysis used.

This consisted in the determination of the Hausmann numbers by the method of Osborne and Harris [1903]; the exact procedure is given in the paper by Knaggs and Schryver [1924, 2].

Unless otherwise stated the hydrolysis of the protein was carried out by 20 % HCl for 20 hours, taking the precaution to hydrolyse immediately with boiling acid [Knaggs, 1923], and to precipitate the basic nitrogen by phosphotungstic acid under exactly the same conditions of temperature, concentration of nitrogen in solution, etc., to eliminate as far as possible those errors which are likely to occur and are fully described by Kernot and Knaggs [1928, 2].

The effect of HCl and NaOH on the precursors of gelatin.

The precursors were treated for 5 days with 0·2 % NaOH or 0·5 % HCl, and the alkali or acid was removed by washing in running water. A part was electrolysed to remove all electrolytes and the gelatin was extracted with water from the remainder, and was then purified by flocculating twice by means of an electric current [Knaggs and Schryver, 1924, 1]. The analytical results are given in Table I.

Table I.

Horn-pith	Amide-N	Humin-N	Diamino-N	Mono-amino-N	Non-amino-N
A. Treated with 0·2 % NaOH for 5 days, well washed and dried	2·08	0·95	24·49	56·60	15·88
A was electrolysed for 10 days	1·97	1·12	22·88	56·24	17·79
Gelatin from *A* twice flocculated	1·30	0·66	24·20	58·64	15·20
B. *A* treated for 5 days with 0·5 % HCl, washed and dried	1·68	0·90	22·45	58·85	16·12
B was electrolysed for 10 days	1·47	1·73	20·71	58·23	17·86
Gelatin from *B* flocculated twice	1·45	1·82	22·94	58·22	15·57
Tendon					
C. Tendon treated with 0·2 % NaOH and electrolysed 10 days	3·51	0·83	25·13	55·18	15·35
Gelatin from *C*	3·02	0·79	29·50	52·12	14·57
D. Tendon treated with 0·5 % HCl and electrolysed 10 days	4·18	0·69	22·30	56·80	16·03
Gelatin from *D*	3·45	0·99	26·00	53·57	15·99

The Hausmann numbers for gelatin extracted from precursors of mammalian origin have already been given in the paper by Knaggs and Schryver [1924, 2]. A conclusion similar to theirs can be drawn regarding the precursor from the figures in Table I, viz. that a precursor treated with alkali gives on hydrolysis a higher percentage of diamino-nitrogen than one treated with acid.

Further, a precursor undergoes some internal structural change under the influence of an electric current: for example, horn-pith treated with either acid or alkali shows a decrease of approximately 2 % in the percentage of diamino-nitrogen, while a slight decrease in the percentage of amide-nitrogen after electrolysis is noticed. The distribution of nitrogen in gelatin from horn-pith differs very slightly from that in its precursor, except that, in the former, the percentage of amide-nitrogen is less, due no doubt to the removal of amino-groups during extraction. On the other hand, tendon gelatin and its precursor show a considerable difference in the percentage of diamino-nitrogen.

Precursors treated with trypsin.

The precursor used above contained elastic fibres (elastin) whose properties and chemical composition are very different from those of gelatin. Elastin is insoluble in most ordinary reagents, and Richards and Gies [1902] showed that on hydrolysis only 3·34 % of the total nitrogen was present as basic nitrogen, and Kossel and Kutscher [1898] obtained only a minute quantity of arginine from it.

The elastin was therefore removed from the tissue, before extracting the gelatin, by digestion with trypsin. Wilson [1920], Marriott [1921] and Rohm and Haas Co [1922, 1923] have shown that when a mammalian skin is digested with trypsin the elastic fibres only are attacked and collagen remains.

Taking advantage of this fact, ossein, ox-tendon, horn-pith, shark-skin and tendon from the head of a sperm-whale, which had been treated for a long period with 0·2 % NaOH, were digested for 20 days with trypsin in sodium carbonate solution of p_H 8. Alkali-treated precursors only were used as it has been shown by Kühne and Ewald [1877] and Reich-Herzberge [1901] that trypsin will attack acid-treated collagen [see also Plimmer, 1912, and Northrop, 1921, 1, 2].

The tissues were well washed in running water and the gelatin was extracted. By this method it was assumed that practically pure collagen was obtained free from ash and elastic fibres. The precursor and the gelatin extracted therefrom were purified as above and the Hausmann numbers of each determined (Table II).

As well as digesting the elastic fibres, the action of trypsin on the precursor produces changes in the collagen molecule. This is shown to be the case by comparing the results given in Tables I and II for horn-pith. Alkali-treated horn-pith has a higher percentage of diamino-nitrogen than that treated with trypsin, the change being from 22·88 to 20·81. The gelatins obtained from each show marked differences, especially in the basic fraction,

Table II. *Hausmann numbers of gelatin and its precursors, the precursors having been treated with trypsin.*

	Amide-N	Humin-N	Diamino-N	Monoamino-N	Nonamino-N
A. Ossein	2·04	2·09	23·66	57·85	14·36
A_1. Gelatin from *A*	2·10	0·85	25·77	59·03	12·25
B. Horn-pith	2·10	5·13	20·81	55·19	16·87
B_1. Gelatin from *B*	1·79	0·98	26·79	58·25	12·19
C. Shark-skin	3·92	1·68	24·60	57·92	11·88
C_1. Gelatin from *C*	3·43	1·41	31·53	46·45	17·18
D. Whale-tendon	3·74	3·88	22·60	64·34	5·44
D_1. Gelatin from *D*	2·88	1·25	24·77	60·85	10·25

which is very much greater when the precursor has been treated with trypsin. In general the action of trypsin on collagen is to increase the percentage of humin-nitrogen at the expense of the diamino-fraction. The Hausmann numbers of the gelatins extracted from enzyme-treated collagens show a normal value for the humin-nitrogen, but the percentage of basic nitrogen is very high. Sadikoff [1903, 1904] showed that gelatins from tendon, previously treated with trypsin and then with various strengths of alkali, differed from each other in physical properties. It is quite possible that these variations were due to the p_H of the solutions of alkali used.

THE VARIATIONS IN THE PHYSICAL PROPERTIES OF GELATINS EXTRACTED FROM A PRECURSOR TREATED WITH HCl OR NaOH SOLUTIONS OF DIFFERENT CONCENTRATIONS.

The following experiment was carried out with the object of trying to find in what way the intramolecular changes in the collagen molecule influenced the physical properties of the gelatin obtained from it.

50 g. of purified horn-pith were treated with 2 litres of 2 % NaOH solution for 5 days. The horn-pith was then thoroughly washed to remove all the alkali, and the gelatin, extracted at 80°, was purified by flocculation as before.

The process was repeated with other samples of horn-pith using 1, 0·5, 0·2 % NaOH solutions, and HCl solutions of the same concentration as those of the alkali. A 5 % solution of each of these samples of gelatin was prepared, and the viscosity at 40° was determined. The drop-number for soya bean oil was also determined by the methods used by Kernot and Knaggs [1928, 2].

Estimations of amino-nitrogen before hydrolysis and of nitrogen precipitated by phosphotungstic acid after hydrolysis were carried out for each sample. The results are given in Table III and shown graphically in Fig. 1.

The curves *A*, *B* and *C* (Fig. 1) for drop-number, percentage of free amino-groups, and of nitrogen precipitated by phosphotungstic acid from the hydrolysed gelatin respectively are roughly of the same shape and follow one another fairly closely. For example, there are maxima around the region of 0·2 % and 1 % on the acid side and 1 % on the alkali side. The minima occur at 1 % acid and 0·5 % alkali. The viscosity curve *D* may roughly be described as the mirror image of *A*, *B* or *C*. In each case the minimum values indicated on the viscosity curve are accompanied by corresponding maximum values on the other curves.

Table III.

Treatment of horn-pith before extraction	p_H	5 % solution: Drop-number for soya bean oil	5 % solution: Viscosity	Gelatin not hydrolysed. Amino-N %	Hydrolysed by 20 % HCl. Diamino-N %*
2·0 % HCl	0·35	64	2·634	4·96	35·43
1·0 % ,,	0·72	59	3·449	4·78	29·4
0·5 % ,,	1·10	61	2·944	4·86	32·3
0·2 % ,,	1·66	64	2·702	4·50	32·2
0·2 % NaOH	12·57	62	6·040	4·31	28·3
0·5 % ,,	13·3	64	4·385	4·38	26·8
1·0 % ,,	13·56	68	2·897	5·27	28·7
2·0 % ,,	13·67	66	4·033	4·78	35·14

* In this case the amide- and humin-nitrogen were not removed before precipitating the basic fraction. The HCl was removed by distillation under reduced pressure, and the residue dissolved in water. The precipitation with phosphotungstic acid was then carried out under the same conditions as described above.

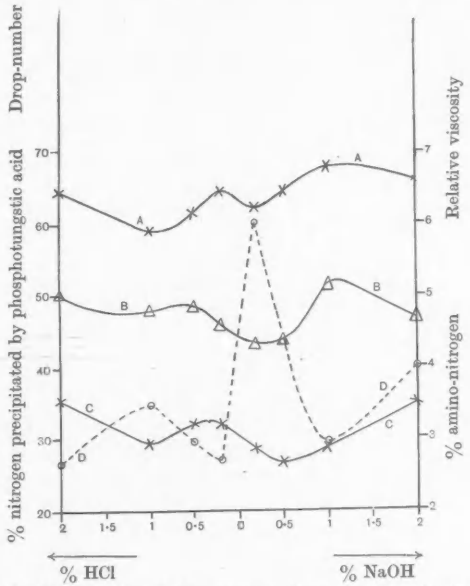


Fig. 1. Final treatment of precursor before extraction of gelatin. Purified gelatin from horn-pith.

A. Drop-number, ×. *B*. % amino-N, △.

C. % N precipitated by phosphotungstic acid after hydrolysis with 20 % HCl, ×.

D. Relative viscosity, ⊙.

It was pointed out by Kernot and Knaggs [1928, 2] that the emulsifying power of a sample of gelatin depended on the previous treatment of the precursor, a gelatin from an alkali-treated precursor being the more efficient, and they concluded that this was due to the higher viscosity and to the presence of more free amino-groups in the gelatin molecule. From the curve in Fig. 1 this conclusion may be extended to include the percentage of basic

nitrogen, which also varies with the drop-number. This is in agreement with the conclusion of Van Slyke and Birchard [1913] who state that the percentage of amino-nitrogen is equal to half that of the lysine-nitrogen in the protein.

It is interesting to note that the changes in the viscosity of gelatin are greatest when the precursor is first treated with acid or alkali and purified before extraction. Schroeder [1903] obtained results of a lower order, owing to the fact that the acid or alkali was not removed from the treated gelatin before the determinations were carried out.

These increases in the viscosity of gelatin due to the effect of acid and alkali have been explained by assuming that the molecules of the protein either form aggregates or become more highly hydrated in these solutions. The enormous increase in the viscosity of gelatin from a precursor treated with alkali, particularly 0·2 % NaOH, cannot be explained solely in this way. It is more likely to be due to the collagen molecule undergoing intramolecular re-arrangement, the nature and degree of such changes having a direct relationship to the strength of the acid or alkali used to treat the precursor.

The relationship between the swelling of a precursor in solutions of NaOH and HCl, and the changes in the chemical constitution which accompany the swelling.

This section is devoted to a study of the changes which occur in collagen when it is treated with solutions of NaOH or HCl. The results in Tables III and IV show that if the NaOH or HCl is not removed before hydrolysis, similar changes to those already mentioned in the Hausmann numbers occur, *i.e.* there is an increase in the percentage of basic nitrogen with both acid and alkali treatment, greater with the latter.

If the precursor is treated as above and hydrolysed with 5 % HCl instead of 20 % HCl other values for the Hausmann numbers are obtained as shown by the results in Tables IV and V for horn-pith and whale-tendon.

Table IV.

Horn-pith	Amide-N	Humin-N	Diamino-N	Mono-amino-N	Non-amino-N
A. Treated with 0·2 % NaOH, washed and dried	2·08	0·95	24·49	56·04	16·44
B. *A* electrolysed	1·97	1·12	22·88	56·24	17·79
B treated with 0·5 % HCl and hydrolysed without washing	1·63	0·38	23·64	59·02	15·33
B treated with 0·5 % NaOH and hydrolysed without washing	1·36	1·51	26·74	52·52	17·87
C. *A* treated with 0·5 % HCl, washed and dried	1·68	0·90	22·45	58·85	16·12
D. *C* electrolysed	1·47	2·73	20·71	57·23	17·86
D treated with 0·5 % HCl and hydrolysed without washing	1·51	0·87	24·07	55·34	18·21
D treated with 0·5 % NaOH and hydrolysed without washing	1·41	1·44	26·16	53·37	17·62

Whale-tendon. The whale-tendon was well washed and cut up into small pieces. It was treated for 10 days with 0·2 % NaOH, the solution being changed daily. It was only necessary to treat the material for a short time with alkali as the amount of chondroitinsulphuric acid present was very much smaller than in ox-tendon. The tendon was well washed, dried, and treated as described below, the Hausmann numbers being determined after each treatment.

Table V.

	Hydrolysed by	Amide-N	Humin-N	Diamino-N	Mono-amino-N	Non-amino-N
E. Dry whale-tendon	20 % HCl	4·23	0·98	25·84	66·12	2·83
E treated 5 days with 0·5 % HCl	20 % "	4·82	2·21	23·86	65·21	3·90
	5 % "	3·84	3·95	27·68	63·59	0·94
E treated 5 days with 0·5 % NaOH	20 % "	3·17	2·11	30·36	58·32	6·04
	5 % "	4·73	4·01	29·82	50·54	10·90

Knaggs [1923] and Kernot and Knaggs [1928, 2] showed that treatment of a protein with cold 20 % or 5 % HCl before hydrolysis caused structural changes in gelatin or its precursors. The above results indicate that 0·5 % HCl and 0·5 % NaOH produce other changes, from which it may be concluded that the nature and degree of the change depend on the strength of the acid or alkali used. This fact was utilised to try to elucidate the changes which occur in tendon and ossein during swelling with different concentrations of HCl or NaOH. The following experiments were therefore carried out, using tendon and ossein.

Swelling of tendon and ossein.

About 1 g. of the precursor was weighed out in a Gooch crucible. The crucible was suspended in a solution of HCl or NaOH of known p_H, contained in a wide-mouthed bottle of approximately 400 cc. capacity. The bottles were kept submerged to the neck in a thermostat at 25° for 48 hours, after which the crucibles were removed and the collagen was rapidly dried with filter paper and weighed. The p_H of the liquid was taken and the amount of nitrogen which diffused from the collagen into the solution was determined in each case by Kjeldahl's method.

For tendon the range of p_H was between 0 and 14, but the swelling of ossein was carried out using solutions of HCl and NaOH above *N* concentration, *i.e.* up to 20 %, because this precursor was treated with similar concentrations of cold acid and alkali before hydrolysis.

The high concentrations of acid or alkali are given as percentages, and the original concentration is used in plotting the curves. The results for swelling and the diffusion of nitrogen are shown graphically in Fig. 2, and these are corrected to 14 % of moisture for each tissue used.

The curves for the change in the swelling of tendon and ossein with p_H are very similar to those reported for other tissues and proteins by other investigators—Kaye and Jordan-Lloyd [1924, 1, 2] for goat-skin, Porter [1921, 1922] for hide-powder, Jordan-Lloyd [1920] for gelatin, Loeb [1920–21] for caseinogen, Fischer and Hooker [1918], Tolman and Stearn [1918] for

blood-fibrin and Jordan-Lloyd [1916] for the sterno-cutaneous muscle of a frog. Their work shows that the maximum swellings occur at an external p_H between 2–3 and 11–12 and the minimum swelling at the isoelectric point. The results for tendon and ossein are very similar to those above, viz. the maximum swelling for tendon on the acid side is at p_H 1·2 for the alkali-treated tissue and at p_H 1·5 for the acid-treated tissue, but no well-defined point for the minimum swelling is observed between p_H 4 and 10 as it varies very little. Tendon shows no maximum on the alkaline side. For ossein the maxima occur at p_H 0·6, 2·2 and 12·1, while the curve is very like that given for goat-skin treated with trypsin by Kaye and Jordan-Lloyd [1924, 2].

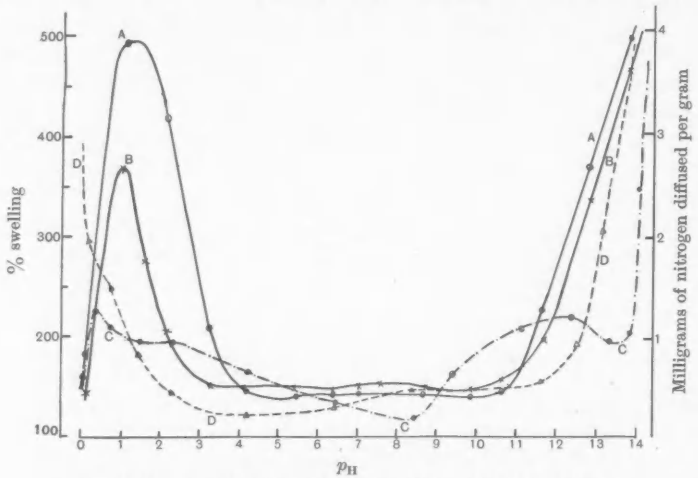


Fig. 2. Swelling of tendon and ossein in HCl and NaOH solutions.

A. Tendons treated with HCl, ⊙.
B. Tendons treated with NaCl, ×.
C. Ossein treated with trypsin, ⊕.
D. Milligrams of nitrogen diffused per gram of ossein, ⊙.

The minimum value for ossein is at p_H 8·5, but there is no minimum shown on the acid side of the curve. The number of readings taken between p_H 4 and 10 does not warrant any definite conclusion being drawn from this fact, but it is interesting to note that Wilson and Kern [1923] claim that ash-free gelatin has a second isoelectric point at p_H 7·7. Thomas and Kelly [1925] hold the same view both for gelatin and collagen (hide-powder). Ossein treated with trypsin may have a different isoelectric point from collagen which has had no such treatment. It was shown by Gerngross and Bach [1923] that the isoelectric point of gelatin varied between p_H 4·45 and 5·55 according to its source. They also showed that treatment with formaldehyde lowered the isoelectric point of one sample of gelatin from p_H 5·05 to 4·6, and with another sample from 4·75 to 4·3. This point about ossein requires further investigation.

At p_H 0 to 4 and 10 to 14 acid-treated tendon swells more than the alkali-treated precursor; but this is reversed between p_H 4 and 10 (see Fig. 2). Bracewell [1919], from a study of the amount of acid absorbed by proteins (gelatin, fibrin, caseinogen, gliadin, edestin), concluded that this absorption is determined mainly by the content of lysine and arginine and is roughly proportional to the number of amino-groups per gram of protein.

No doubt this is the case for collagen at low concentrations of acid and alkali. By analogy with gelatin the alkali-treated tendon would swell more than that treated with acid, which is in agreement with the curves *A* and *B*, Fig. 2, but only between p_H 4 and 10. At higher concentrations of acid and alkali other factors come into play which considerably alter the degree of swelling of tendon.

Unfortunately there is no reliable method for the direct determination of the free amino-groups in collagen on account of its insolubility.

Between p_H 1 and 13 the amount of nitrogen which goes into solution is very small and, from Fig. 3 for ossein, it will be noticed that the collagen begins to disperse only at high concentrations of acid or alkali.

The hydrolysis of ossein and tendon.

The behaviour of tendon and ossein near the points of maximum swelling is not properly understood, but there is no doubt that the protein undergoes internal structural changes, the first result of which is the swelling and the second the dispersion of the tissue into solution. The following experiments were carried out with tendon and ossein to try to elucidate the changes which occur.

Tendon. The acid- and alkali-treated tendons were kept for 4 days in the thermostat at 20° with solutions of NaOH and HCl of concentrations between 0·125 % and 1 % and a blank was carried out, using boiled distilled water.

The tendon was quickly removed from the liquid, thrown into boiling 20 % HCl, and the heating continued for 20 hours. The Hausmann numbers of the hydrolysate were determined, taking the precautions mentioned above. The basic nitrogen was always precipitated from 100 cc. of solution which contained approximately 180 mg. of nitrogen.

The results obtained are plotted in Fig. 3 and on the same curve, for comparison, are included the percentage swellings of tendon, taken from Fig. 2.

The treatment of tendon with cold solutions of NaOH or HCl, followed by immediate hydrolysis with hot 20 % HCl, alters the distribution of nitrogen in a similar way to that observed with horn-pith.

Diamino-nitrogen. The action of cold NaOH on tendon is to increase the basic nitrogen while that of cold HCl decreases this value. It will be noticed that curves *A* and *B* (Fig. 3) are very similar in shape.

Nonamino-nitrogen. As this value is obtained by difference, no satisfactory conclusions can be drawn from the numbers unless there are large variations.

This is the case with alkali-treated tendon, there being a gradual decrease with cold HCl, while with cold NaOH the percentage of nonamino-nitrogen decreases rapidly as the concentration of NaOH increases, due probably to the ring compounds being converted into open chain compounds by the action of the alkali. With the acid-treated precursor, the nonamino-nitrogen increases with either HCl or NaOH but the changes are not large.

These differences in behaviour must be due to the different molecular structure of the two precursors.

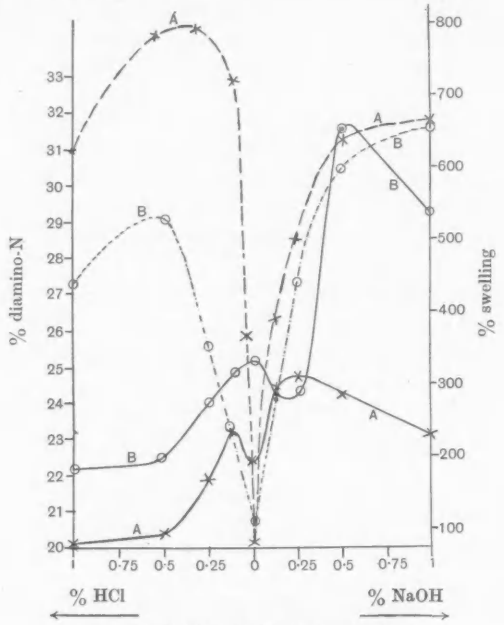


Fig. 3. Achilles tendon (ox). Air dried.

A. Final treatment with HCl solution, ×.

B. Final treatment with NaOH solution, ⊙.

——— Diamino-N after hydrolysis with 20 % HCl.

— — — Swelling, *A*. —•—•— Swelling, *B*.

Swelling of the tendon.

The points of inflection on the curves *A* and *B* (Fig. 3) for swelling and percentage of diamino-nitrogen are given in Table VI.

The results obtained indicate that, when a tissue swells in acid, the internal structure of its molecule is altered, and, generally, as the concentration of the acid is increased the percentage of diamino-nitrogen decreases. At the point of maximum swelling the percentage of basic nitrogen is a minimum. With alkali the diamino-nitrogen increases, and it follows that, at the point of maximum swelling, the percentage of diamino-nitrogen is also a maximum.

Table VI.

Alkali-treated tendon				Acid-treated tendon			
Diamino N %		Swelling		Diamino-N %		Swelling	
Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.
0	0·5 % HCl	0·4 % HCl	0	0·125 % HCl	0·5 % HCl	0·5 % HCl	0
0·5 % NaOH	0·25 % NaOH	0·75 % NaOH	0	0·25 % NaOH	0	0·75 % NaOH	0

Hydrolysis of ossein free from elastin.

An experiment similar to the above was carried out, using ossein from which the elastin had been removed by trypsin. In this case the range of concentrations of the acid and alkali used was greater, from 20 % to 0·66 %.

10 g. of ossein were treated with 300 cc. of 0·66 % HCl for 4 days at 20°; the supernatant liquid and a part of the protein were heated for 20 hours at the boiling-point, and the remainder was hydrolysed with 20 % HCl (300 cc.). This was repeated, using the other concentrations of HCl and NaOH given in Fig. 3.

With 15 % and 20 % HCl and NaOH, a large part of the ossein dissolved, so that only the liquid was heated, and the undissolved part was hydrolysed by 20 % HCl.

Two sets of results were thus obtained (Fig. 4), one in which the ossein had been treated throughout the experiment with the same strength of acid or alkali, and another where the ossein, after treatment with cold acid or alkali, was hydrolysed by 20 % HCl.

Hydrolysis of ossein after treatment with cold HCl or NaOH.

(*a*) *With* 20 % *HCl.* The products of hydrolysis of ossein vary considerably according to the previous treatment, in a similar way to that observed with tendon and horn-pith. The amide-nitrogen varies slightly. An increase is noticed on the acid and a slight decrease on the alkaline side. With 20 % NaOH the percentage falls to 0·8 showing that only very concentrated solutions of alkali hydrolyse the free amino-groups in the cold.

The percentage of diamino-nitrogen is plotted against the concentration of acid or alkali used in treating the ossein before hydrolysis in Fig. 4. It is noticed that there is a minimum value for 10 % HCl, and the curve ascends rapidly as the strength of acid is increased or decreased, the maximum value being with 2 % HCl.

There is a second minimum at zero which corresponds to the ossein which had been treated with water only.

On the alkaline side there is one maximum at 2·5 % NaOH.

(*b*) *With the same strength of acid or alkali with which the ossein was treated in the cold.* Referring to the curves in Fig. 4, it is noticed that all three curves are similar in form; for example, considering the curves *A* and *B*, there

is a fairly constant difference between them, and it is only near the isoelectric point that this difference diminishes.

The zero point for curve *A* is not theoretically correct and is obtained by continuing the two branches of curve *A*. Theoretically this should be 100, on the assumption that when pure collagen is treated for 4 days with pure water and then heated for 20 hours no hydrolysis occurs and the whole of the protein is therefore precipitated by phosphotungstic acid.

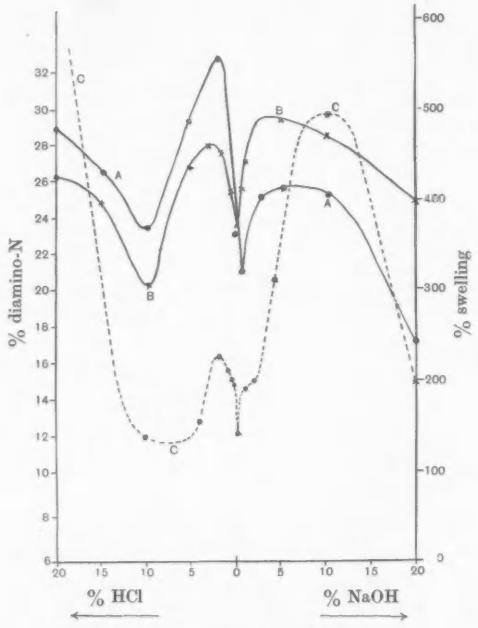


Fig. 4. Ossein (free from elastin).

A. Percentage of diamino-N, after standing in contact with HCl and NaOH of different strengths, indicated on abscissae, and hydrolysed with acid or alkali of the same concentration.

B. Percentage of diamino-N, after standing as for *A*, but ossein hydrolysed with 20 % HCl.

C. Swelling in acid and alkali of different concentrations.

The results for the hydrolysis of ossein with different concentrations of HCl are somewhat similar to those obtained by using 20 % HCl, the chief differences being in the higher percentage of basic nitrogen, and in the percentage of nonamino-nitrogen decreasing as the concentration of acid is increased.

The hydrolysis with NaOH is very different, the final products no doubt being different. The percentage of diamino-nitrogen is less than that obtained when 20 % HCl is used for hydrolysis. There is a loss of nitrogen as ammonia, due to the scission of some of the amino-groups, especially at the higher

concentrations of NaOH, and during hydrolysis an insoluble nitrogen compound is formed. This was removed by filtration before the Hausmann numbers were determined.

Fosse [1912] showed that when a protein was hydrolysed by alkalis, the arginine was split into urea and ornithine. The former would be decomposed by the alkali into ammonia, while the latter would be precipitated by phosphotungstic acid. Ornithine contains a lower percentage of nitrogen than arginine, and in consequence the percentage of basic nitrogen would be lower, which is in agreement with the results (Table X).

As stated above the effect of cold NaOH appears to cause scission of the rings of closed chain compounds. This effect is more marked with hot alkali, for it is noticed that the percentage of nonamino-nitrogen decreases gradually as the concentration of the NaOH is increased, until with 20 % NaOH it is reduced to zero.

The relationship between the degree of swelling and the products of hydrolysis of tendon and ossein.

On comparing the curves in Figs. 3 and 4 for the percentage of diamino-nitrogen of ossein and tendon after hydrolysis, using 20 % HCl, it is noticed that they resemble one another in shape although the range of concentration of acid and alkali used for tendon is not so great. Tendon is much more sensitive to a small change in p_H than ossein, especially on the acid side. A striking point about the swelling curve for ossein is that, at the point of maximum swelling, the percentage of diamino-nitrogen is a maximum on the acid side (with tendon this is a minimum), but, on the alkaline side, the highest value for the basic nitrogen is at the point on the curve where the swelling begins to increase very rapidly. The percentage of diamino-nitrogen then decreases gradually. At the maximum swelling there is a tendency for some of the protein to become soluble, and it may be that these changes in the distribution of nitrogen in the collagen molecule result in making the protein soluble in HCl or NaOH solutions.

Hydrolysis of fibrin from ox-blood.

An experiment similar to that just described for ossein was carried out, using fibrin from ox-blood. The fibrin was prepared as follows [see Hammarsten and Hedin, 1924]. The freshly drawn blood was thoroughly beaten and the elastic fibrous masses were well washed in running water. It was obtained free from blood corpuscles, etc. by washing several times with 5 % sodium chloride solution and finally with water. The fibrin was dried by blotting paper, and the following results were obtained (Fig. 5). The solution from which the diamino-nitrogen was precipitated by phosphotungstic acid contained 75 mg. of nitrogen (approximately).

No results are given for the swelling of fibrin, as this substance easily

disintegrates in strong solutions of HCl, and readily dissolves in a solution of NaOH.

The results for the percentage of diamino-nitrogen are shown in Fig. 5, and the curves resemble those for tendon and ossein (Figs. 3 and 4).

It will be noticed that in the curve *B* for hydrolysis, using 20 % HCl, there are maxima at 2·5 % HCl and 1 % NaOH and minima at 10 % HCl, 0·5 % and 3·3 % NaOH, and for curve *A* similar points of inflection occur at the same concentrations except on the acid side, when the maximum is at 0·66 % HCl, being no doubt due to incomplete hydrolysis.

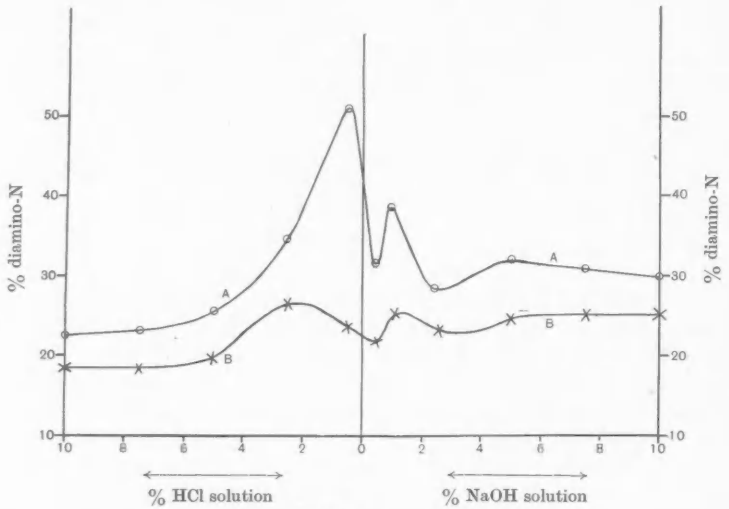


Fig. 5. Fibrin from ox-blood.

A. In contact with a solution of HCl or NaOH of varying strength for 4 days at 20° and hydrolysed at 100° with same concentration of acid or alkali, ⊙.

B. Same as *A* but hydrolysed at 100° with 20 % HCl, ×.

Curves *A* and *B* (Fig. 5) do not cross, as they do in the case of ossein (Fig. 4). Curve *A* is always above *B* even when NaOH is used as the hydrolytic agent. This may be due to the relative amounts of lysine, arginine and histidine being different in ossein and fibrin. The analytical results obtained by several investigators are given in Tables VII and VIII.

Table VII.

	Arginine	Histidine	Lysine	Observer
Fibrin	10·91	4·36	12·05	Gortner [1916]
Fibrin	13·14	3·96	11·4	Van Slyke [1911]
Gelatin	14·0	3·59	6·20	Van Slyke [1911]

The results given in Table VII were obtained, using the method of Van Slyke [1911] for the estimation of the distribution of nitrogen in a protein.

Fibrin contains a lower percentage of arginine and a much higher percentage of lysine than gelatin, and also in this case the total diamino-nitrogen is greater.

Other workers obtained the following results by isolating the individual amino-acids after hydrolysis of the protein.

Table VIII.

	Arginine %	Histidine %	Lysine %	Observer
Fibrin	4·61	2·05	5·18	Lock and Thomas [1913]
,,	3		4	Kutscher [1898]
Gelatin	7·62	0·40	2·75	Hart [1901]
,,	8·2	0·9	5·9	Dakin [1920]

It is seen that the results for both gelatin and fibrin do not always agree. This may be due to the previous treatment these proteins received before hydrolysis, which probably altered appreciably their chemical constitution. It may be concluded from these results (Tables VII and VIII) that fibrin contains a much lower percentage of arginine than does gelatin, and that the decrease in the basic fraction when gelatin is hydrolysed by sodium hydroxide is due to the decomposition of the arginine into urea and orinthine.

Fibrin contains a much higher amount of amide-nitrogen than does gelatin, and consequently any changes which occur are more readily detected with this protein. It will be noticed that an increase in the concentration of NaOH causes the percentage of amide-nitrogen to diminish, and, whatever the concentration of alkali used, this value is always much less than when acid is used for the hydrolysis.

The low numbers obtained, using 1 % or 0·5 % HCl, are due to incomplete hydrolysis of the protein.

When fibrin previously treated with cold alkali is hydrolysed by 20 % HCl, it has a much higher percentage of amide-nitrogen than in the other cases, in this respect resembling collagen.

Discussion.

From the foregoing results it may be concluded that collagen, when treated with cold solutions of HCl or NaOH of different concentrations, undergoes internal structural changes especially at p_H 2 or less and p_H 11 or more, *i.e.* at and above the points of maximum swelling. Between p_H 3 and 10 the chief reaction which takes place is that the acid or alkali combines with the free amino- and carboxyl-groups, forming salts. At other concentrations free acid or alkali is present which probably brings about intramolecular change.

It is a well-known fact that, if the chemical constitution of a compound is altered, such changes are accompanied by variations in the physical properties of the substance. This may be attributed in the case of proteins primarily to salt formation and secondly to spatial re-arrangement of the atoms composing the protein molecule caused by the action of the acid or alkali.

These tautomeric changes in collagen take place slowly and the compounds formed are fairly stable. Dakin [1912] showed this to be the case from a study of the changes with time in the optical rotation of gelatin in alkaline solution.

The Hausmann numbers indicate that the action of cold HCl and NaOH on collagen is to produce fairly stable compounds whose molecules, on hydrolysis with boiling 20 % HCl, undergo scission at different points in the chain to yield different end-products. This is possibly due to the peptide linkages existing in two tautomeric forms, which break down in different ways on hydrolysis.

The evidence in favour of two or more isomers of collagen is as follows.

(1) The Hausmann numbers of acid-treated collagen differ in the percentages of amide-nitrogen and diamino-nitrogen from those of an alkali-treated collagen. The gelatins extracted from these precursors also differ in a similar way.

(2) The percentage swelling of collagen in HCl and NaOH of varying p_H values differs according to the previous treatment.

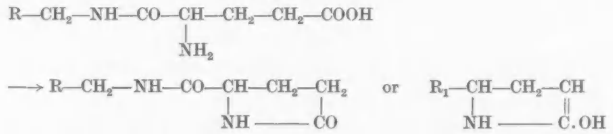
(3) The rate of extraction of gelatin from an acid-treated precursor is greater than from the same precursor which has had alkali treatment [Knaggs and Schryver, 1924, 2].

(4) Gelatin from an alkali-treated precursor has a higher percentage of free amino-groups than gelatin from an acid-treated precursor. The former has a higher viscosity and emulsifies animal or vegetable oils more easily than the latter [Kernot and Knaggs, 1928, 1].

(5) Treatment of acid- or alkali-treated collagen with cold solutions of HCl or NaOH of different concentrations and subsequent hydrolysis with 20 % HCl gives different hydrolysis products. The greatest variation is noticed in the percentage of basic nitrogen. A gelatin from alkali-treated collagen has a higher percentage of free amino-groups than one from a precursor treated with acid.

An explanation of the above is very difficult owing to the complex structure of a protein molecule, but several useful facts may be deduced.

On repeated flocculation of gelatin in an electric field or on heating the solution of ash-free gelatin, an increase in the percentage of nonamino-nitrogen is shown [Knaggs and Schryver, 1924, 3]. This is probably due to the glutamic acid residue which [see Dakin, 1912] occupied a position at the end of a branched chain, forming a lactam.



Such a group could not be estimated by the Van Slyke method.

Collagen does not behave in this way, as the percentage of nonamino-nitrogen does not increase when it is electrolysed for several days. It may

be assumed from this that probably the branched chains of gelatin are formed into rings in collagen [Procter, 1917; Procter and Wilson, 1923] and these rings are ruptured when the gelatin is extracted by hot water. Treatment with cold solutions of acid or alkali probably has the same effect.

This will no doubt explain the difference in the degree of swelling of gelatin and collagen. Kaye and Jordan-Lloyd [1924, 2] state that the volumes in cc. occupied after swelling by 100 g. vacuum-dried material at 20°, at p_H 2·3 for powdered gelatin and hide-powder are 11,550 and 3700 respectively and 2700 and 2100 respectively at p_H 5·1.

By using hide-powder the restraining influence on swelling, due to the fibrous structure of the skin, is removed and therefore the above numbers are comparable.

At the point of maximum swelling gelatin swells considerably more than collagen; this may be explained by assuming that the collagen molecule is mainly composed of closed rings, whereas in gelatin some of these rings are open. The acid or alkali forms salts and the evidence favours the view that these salts are highly hydrated [Pauli, 1922; Hardy, 1905].

The experimental results above seem to indicate that at the point of maximum swelling the rings of additional closed chain compounds are ruptured, which gives further points of attachment for the acid or alkali, and consequently an enormous increase in the hydration of the protein molecule. This is shown by the greater amount of the acid absorbed by gelatin [Jordan-Lloyd and Mayes, 1922], and also by the fact that, if a protein is treated with concentrated solutions of acid or alkali, it cannot be recovered again in the original condition.

Summary.

1. Samples of collagen and the gelatin extracted therefrom, derived from different mammalian tissues, were prepared in a highly purified state.

2. The collagens were treated with HCl and NaOH of varying concentrations for known periods. It was found that an alkali-treated precursor gave on hydrolysis a higher percentage of diamino-nitrogen than one treated with acid.

3. Ash-free gelatins were prepared from horn-piths differently treated. Curves are given showing how the previous treatment of the precursor affects the viscosity, drop-number and percentages of amino- and diamino-nitrogen. The viscosity curve is roughly the mirror image of the other three curves.

4. The elastin was removed from collagen by tryptic digestion, and the resulting product on analysis gave results which seem to indicate that the enzyme produces intramolecular changes in the protein molecule.

5. The swelling of collagen in HCl and NaOH of different p_H values was carried out. The hydrolysis of the swollen tissues was studied. The following conclusions were drawn from the results.

(*a*) The previous treatment affects the degree of swelling.

(*b*) Around the point of maximum swelling the percentage of diamino-nitrogen is either a maximum or a minimum. Therefore it is concluded that swelling is accompanied by internal structural changes and scission of closed rings. The latter gives rise to more free groups for the attachment of molecules of water.

(*c*) Collagen prepared from different tissues has not the same chemical constitution.

I wish to say how very grateful I am for the advice and help given me by the late Prof. Schryver during the course of this work.

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CXLV. THE INFLUENCE OF PROLONGED MUSCULAR REST ON METABOLISM.

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(*Received October 23rd, 1929.*)

Whereas an extensive literature has grown around the question of the influence of work on metabolism, there is one phase of metabolism which has received but little attention, namely, the metabolic changes produced by prolonged muscle rest. This was specially brought home to the present writer while investigating the changes occurring in the mineral metabolism during the healing of fractured bones. It was noted that while there was usually a slight negative balance of calcium, there was an excessive loss of phosphorus, which, during the second week of healing, for example, amounted in some cases to an average of 2·6 g. P_2O_5 daily. Was this excessive loss of phosphorus the result of a local mineral change at the site of fracture, involving a retention of calcium and loss of phosphorus, or was it, on the other hand, the result of the wasting or spasm of muscle, either alone, or accompanied by an atrophy of bone? Determinations of the nitrogen balance further revealed that there was an excessive loss of this element, and it was obvious that before one could attempt to interpret these changes in patients suffering from fractures, it was necessary to gain some insight into the more fundamental problem of the disturbed metabolism produced by prolonged rest.

Although the maintenance of normal functional activity necessitates a balance between anabolism and catabolism it is, however, almost impossible to define what this normal balance is. Different individuals who resemble each other in muscular build may have very different levels of functional activity, which they term their normal routine. The experiments which are to be described were designed to produce such a lowering of muscular metabolism as to be definitely below any arbitrary "normal." They are concerned with pure disuse atrophy, and not with atrophy due to destructive lesions of the lower motor neurons or their axons, or to toxic action, or to pressure. It has long been observed that muscular wasting associated with bone or joint disease is more rapid and extreme than that which occurs with simple disuse of a limb. In the former, visible loss of substance may be seen in a day or two. It is quite definite also, that there is a more rapid and extreme wasting in limbs immobilised for some inflammatory or traumatic lesion,

than in limbs immobilised to correct a deformity. Harding [1925, 1926, 1929] has thrown some light on this problem. She found that the oxygen consumption of muscles wasted as the result of arthritis is definitely increased above normal; whereas that of muscles wasted from pure disuse is unaltered. Further, that to produce the first type of atrophy experimentally, there must be complete integrity of the afferent nervous paths, and that the atrophy of pure disuse can be still produced even after de-afferentation at the posterior columns. There appear then to be two quite distinct types of atrophy—one which is mainly of reflex origin, and one which is probably entirely due to imposed rest.

Observations such as those of Ollerenshaw [1925] and Smith [1928] throw considerable doubt on the adequacy of our conceptions of the mechanism of disuse. These authors describe three cases of congenital absence of the tibia, where the *tibiofibularis* muscle had only one attachment. During life they subserved no function, and were unable to shorten; nevertheless they persisted without atrophy or degeneration. The oldest subject was 13 years old at the time of amputation. On the other hand, Lipschütz and Audova [1921] state that section of the *tendo Achillis* in rabbits causes an atrophy of muscle which is nearly as great as that caused by nerve sections. They believe that atrophy is due to a great decrease of work performed. Incisions of muscle and tendon without complete section did not produce such pronounced effects.

On reviewing the literature on the chemical side, it is surprising to find how little this aspect of metabolism has been studied. As early as 1855 [Beigel, 1855] had compared the urinary excretions of four healthy subjects during periods of active recreation and of comparative rest. The average daily output of urea during the period of active movement was 52·26 g., and during the period of rest on a sofa 46·10 g. Shaffer [1908] made observations on a man who spent 2 days wholly in bed, and then on the following 4 days got up for a few hours to sit in a chair. The rest was sufficient to weaken the patient noticeably. This period was followed by one of normal activity. The average total daily nitrogen excretion was 4·77 g. and 4·40 g., while the sulphur excretion was 0·438 g. and 0·424 g. respectively, during these two periods. There does not appear to have been any pre-rest period to ensure that nitrogen equilibrium was established. Shaffer, however, concludes "that either increase or decrease of muscular activity within physiological limits has *per se* no effect on protein metabolism as indicated by the urinary excretion of nitrogen and sulphur."

That the problem is complicated by an atrophy of bone has been shown by Allison and Brooks [1921, 1922], who found that the amount of bony change varied with the degree of disuse, the cause of the disuse being immaterial.

General plan of the experiments.

The subjects of the present series of experiments were either volunteers in perfect health, or were patients admitted to hospital with loose fragments of cartilage in the knee-joint. These latter, apart from occasional locking of the knee-joints, suffered no discomfort, and were in good health. They were allowed to select the quality and quantity of foodstuffs they desired from a prepared list. This intake, as well as the intake of water, was kept constant during the course of the experiment, except where expressly stated. The urine was collected in 24-hourly specimens, made up to a constant volume, and preserved with thymol in chloroform. The various periods during which faeces were collected were marked off with carmine, taken in the form of capsules. The faeces were dried on a steam-bath and in an air-oven before being stored in sealed bottles. A pre-period of 4 to 5 days was usually allowed in order to obtain nitrogenous equilibrium. During this pre-rest period, the subjects led a sedentary life, performing the ordinary routine of the ward. In Exp. 1, however, the level of activity, though higher, was still very constant. When nitrogenous equilibrium was obtained, the subject was confined to bed, with one lower limb encased in a well-padded osteotomy splint, the foot-piece being anchored. In some of the experiments, the activity of the other leg was limited by being loosely attached to a sand-bag. No massage was allowed, and only occasionally were the splints readjusted. The subjects were propped up in bed, and asked to limit, as far as possible, all superfluous movements.

Analytical methods.

(*a*) *Urine:*

Total acidity:	Expressed as cc. 0·1 *N* acid in 24 hrs.
Total nitrogen:	Kjeldahl method.
Urea:	Urease method.
Ammonia and creatinine:	Folin's method.
Uric acid:	Benedict and Franke's method [1922].
Total sulphur:	Denis's modification of Benedict's method.
Inorganic sulphur and ethereal sulphur:	Folin's method.
Neutral sulphur:	By difference.
Total phosphorus:	Neumann's wet ash method.
Calcium and magnesium:	Shohl and Pedley's method [1922].

(*b*) *Faeces and food:*

Total nitrogen:	Kjeldahl method.
Total sulphur:	Material extracted and evaporated to dryness with HNO_3 and then with HCl, then Denis's modification of Benedict's method applied.

Phosphorus:	Material ashed in the presence of excess $Ca(OH)_2$: phosphorus precipitated as ammonium phosphomolybdate and weighed directly.
Calcium:	Material ashed, any iron removed, then precipitated according to Shohl and Pedley's method.
Magnesium:	Shohl and Pedley's method.

Exp. 1. The subject, G.P., a university student in perfect condition, offered his services for experimentation. During the first 5 days, he maintained the same level of activity, 1 hour a day being spent walking rapidly in the open. On the fifth day of diet he was confined to bed, with his left leg in a long osteotomy splint, the foot piece being anchored. The right leg was held down by being loosely attached to a sand-bag. The subject lay thus in bed for 11 days.

In addition to the ordinary routine of the ward, on the first day of resumed activity he walked as briskly as possible for 1½ hours; on the second for 2½ hours; for 2¾ hours on the third; 1½ hours on the fourth; and on the last day, approximately 16 miles were accomplished in the first 6 hours of the 24.

During the experimental period the patient's diet was constant. But on the second day of resumed activity, his fluid intake was increased by 200 cc. water, and on the fourth and fifth day, by 400 cc. each day.

Age 19 years; height 181 cm.; weight prior to imposed rest 71·52 kg.; weight immediately after imposed rest 68·66 kg.; weight after 4 days' resumed activity 68·20 kg.

Daily intake of food:

Dried milk:	55 g. (made up in fluid form from Cow and Gate half cream dried milk to 630 cc.).
Brown bread:	215 g.
Digestive biscuits:	90 g.
Fresh butter:	70 g.
Steak:	150 g. (served as stew).
Dried apples:	15 g. (served stewed).
Dried tomato soup:	15 g. (served as soup).
Dried egg:	9 g. (served as custard).
Tea:	400 cc.
Sugar:	25 g.
Marmalade:	1 level teaspoonful.
Juice of 1 orange.	

(Owing to an unavoidable delay in this experiment, a deposition of phosphates had occurred in the urines, before the phosphorus could be estimated. The urinary phosphate figures are therefore omitted.)

Exp. 2. The subject, J.M., a coal-miner, was in good health, and apart from a loose piece of cartilage in one knee, was physically sound. He walked

Table I. (*Exp.* 1.)

State	Vol. of urine cc.	Nitrogen g. Total	Uric acid	Creatinine	Faeces (daily av.)	Sulphur (SO_3) g. Total	Inorganic	Ethereal	Neutral	P_2O_5 g. Faeces (daily av.)	Calcium CaO g. Urine (daily av.)	Faeces (daily av.)
A	1190	12·32	—	—		—	—	—	—			
A	1160	13·44	—	—		—	—	—	—			
A	1040	12·54	0·347	0·651		2·09	—	—	—			
A	1080	12·51	0·335	0·654	1·148	2·08	—	—	—	0·897	0·326	0·694
A	1200	12·40	0·369	0·588	**19·79**	2·08	1·537	0·175	0·372			
1 R	1455	11·31	0·377	0·678		1·82	—	—	—			
2 R	1620	11·46	0·410	0·674		2·04	—	—	—			
3 R	1140	11·40	0·346	0·688		2·03	—	—	—			
4 R	1220	12·77	0·362	0·678		2·22	—	—	—			
5 R	1210	14·00	0·382	0·481		2·33	—	—	—	0·894	0·388	0·809
6 R	1200	14·22	0·389	0·617	1·160	2·26	—	—	—			
7 R	1560	15·45	0·413	0·678	**19·18**	2·48	—	—	—			
8 R	1400	13·66	0·394	0·708		2·32	—	—	—			
9 R	1590	14·78	0·406	0·698		2·46	—	—	—			
10 R	1670	14·24	0·407	0·700		2·38	—	—	—		0·438	
11 R	1580	14·78	0·369	0·625		2·52	2·000	0·135	0·390	0·960		0·957
1 A	1400	12·21	0·353	0·678	1·185	2·45	—	—	—			
2 A	1280	15·01	0·373	0·688	**19·51**	2·56	—	—	—			
3 A	1340	12·87	0·335	0·676		2·25	—	—	—		0·365	
4 A	1300	13·77	0·368	0·674		2·50	—	—	—			
5 A	1320	13·60	0·362	0·678		2·51	—	—	—			
Intake		13·51				2·22					1·212	

Average daily excretion of faecal solids in heavy type under value for average daily excretion of faecal nitrogen for same period.

A = active state. R = resting state.

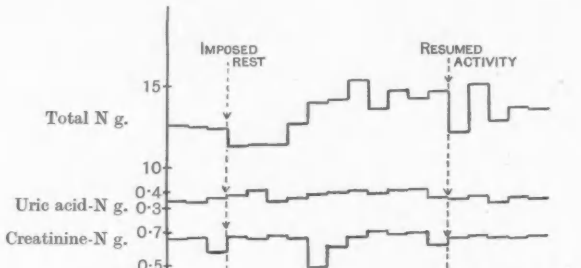

Fig. 1. Experiment 1.

about the ward till the fifth day, when he was confined to bed with an osteotomy splint, extending to a few inches above the knee. From the ninth day of rest onwards, an orange was added to the diet. On the tenth day, castor oil was given in preparation for the operation, which, however, was not performed till the thirteenth day. Stovaine spinal anaesthesia was used. From

the day of the operation till the twelfth day thereafter, the patient lay quietly in bed, his left leg kept at rest between two sand pillows. The operation clips were removed on the tenth day. On the thirteenth day after operation the patient was allowed up for 1 hour, and on the days following for longer intervals. On the third day of resumed activity, it was noticed that a small haematoma had formed in the wound, the bandages being stained with blood.

Age 28 years; height 182 cm.; weight 66·7 kg. at start.

Daily intake of food:

Dried milk:	105 g.	Dried lentil soup:	15 g.
Brown bread:	200 g.	Dried egg:	9 g.
Digestive biscuits:	90 g.	Tea:	400 cc.
Fresh butter:	70 g.	Sugar:	25 g.
Steak:	100 g.	Juice of 1 orange.	
Dried apples:	15 g.	Marmalade:	1 level teaspoonful.

Table II. (*Exp.* 2.)

		Nitrogen g.					Sulphur (SO_3) g.				P_2O_5 g.		Calcium CaO g.	
State	Vol. of urine cc.	Total	Urea	Ammonia	Creatinine	Faeces (daily av.)	Total	Inorganic	Ethereal	Neutral	Urine	Faeces (daily av.)	Urine (daily av.)	Faeces (daily av.)
A	1050	11·87	—	0·480	0·561		2·05	—	—	—	2·17			
A	1100	11·93	9·72	0·431	0·541		1·94	1·41	0·19	0·34	2·57			
1 R	1420	10·41	8·25	0·440	0·488		1·83	—	—	—	1·99			
2 R	1005	11·81	9·42	0·482	0·611		2·19	—	—	—	2·37			
3 R	1020	15·00	—	—	0·669	1·276	2·47	—	—	—	2·93	1·18	0·374	1·649
4 R	950	13·32	—	—	0·558		2·27	—	—	—	2·81			
5 R	1450	12·76	—	—	0·502		2·08	—	—	—	2·65			
6 R	1000	12·20	—	—	0·511		2·10	—	—	—	2·98			
7 R	1500	14·44	—	—	0·539		2·33	—	—	—	3·49			
8 R	1380	13·75	—	—	0·578		2·41	—	—	—	2·83			
9 R	966	14·00	—	—	0·542		2·39	—	—	—	3·09			
Castor oil														
A	840	12·54	—	—	0·561		2·23	—	—	—	3·41			
A	1080	14·00	—	—	0·589		2·45	—	—	—	2·49			
1 R	1200	12·88	—	—	0·545		2·28	—	—	—	2·47			
2 R	1450	13·88	—	—	0·601		2·51	—	—	—	2·57			
3 R	1000	13·87	—	—	0·609		2·31	—	—	—	2·77			
4 R	1220	13·66	—	—	0·600		2·47	—	—	—	2·01			
5 R	1175	15·23	—	—	0·641		2·94	—	—	—	2·95			
6 R	1040	12·43	—	—	0·365		2·18	—	—	—	2·45			
7 R	1580	14·56	—	—	0·700		3·26	—	—	—	3·79			
8 R	1660	17·92	—	—	0·657		2·94	—	—	—	3·95			
9 R	1760	18·27	—	—	0·610		2·99	—	—	—	3·32			
10 R	1830	19·32	—	—	0·548		3·32	—	—	—	3·33			
11 R	1890	19·60	—	—	0·532		3·41	—	—	—	3·65			
12 R	1930	20·14	—	—	0·600		3·65	—	—	—	3·89			
1 A	1970	20·14	1648	0·709	0·638		3·66	—	—	—	3·83			
2 A	1630	21·42	1820	0·841	0·630		3·81	2·96	0·13	0·72	3·51			
3 A	1735	22·54	1840	1·031	0·709		3·91	3·21	0·15	0·70	3·90			
4 A	1560	17·36	1525	1·052	0·591		2·97	—	—	—	2·91			
Intake		13·29					2·20				3·76		1·917	

Exp. 3. This man, J.C., a "jack of all trades," who, 2½ months previously, had had a loose body removed from his knee, and had completely recovered, offered himself as a subject. The experimental period had to be abandoned on the sixth day of imposed rest. The diet was similar in quality and quantity to that described in Exp. 2.

Age 40 years; height 167 cm.; weight 68·2 kg. at start.

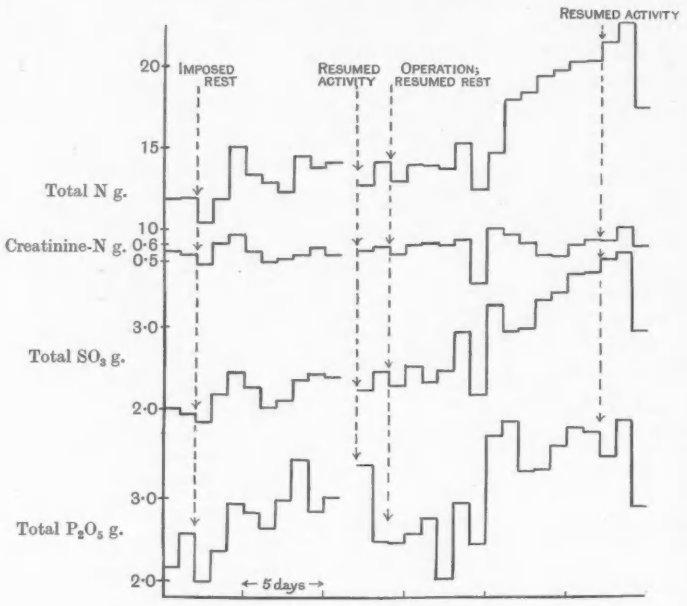

Fig. 2. Experiment 2.

Table III. (*Exp.* 3.)

State	Volume of urine cc.	Nitrogen g.		Sulphur SO_3 g.			
		Total	Creatinine	Total	Inorganic	Ethereal	Neutral
A	—	9·57	—	—	—	—	—
A	1045	12·97	0·44	2·18	—	—	—
A	980	11·34	0·44	1·93	1·56	0·17	0·20
1 R	1310	10·77	0·44	1·79	—	—	—
2 R	—	8·94	0·41	1·50	—	—	—
3 R	1420	11·73	0·57	1·99	—	—	—
4 R	1360	12·35	0·41	2·06	—	—	—
5 R	1400	12·72	0·44	2·10	1·67	0·24	0·19
6 R	1410	13·86	0·53	2·36	1·70	0·25	0·41
Intake		13·29		2·20			

Exp. 4. The subject, J.C., was the same as in Exp. 3, and the procedure was similar to that adopted in that experiment. With the exception of dried lentil soup, which was substituted for tomato soup, the diet was identical. The time interval between Exps. 3 and 4 was 1 month.

Exp. 5. This female subject, E.M., a cook, had been confined to bed at home for a week prior to admission, with a partially loose fragment of semilunar cartilage in her knee-joint. The knee was slightly swollen, otherwise she was in good health. For the first 4 days of residence in hospital she walked a little about the ward, after which she was confined to bed, with a splint encasing her leg. The patient commenced to menstruate on the fourth day after the completion of the experimental period.

Table IV. (*Exp.* 4.)

State	Titratable acidity *N*/10 cc.	Nitrogen g. Total	Urea	Ammonia	Creatinine	Faeces (daily av.)	Sulphur (SO_3) g. Total	Inorganic	Ethereal	Neutral	P_2O_5 g. Urine	Faeces (daily av.)	Calcium CaO g. Urine (daily av.)	Faeces (daily av.)
A	—	12·09	—	—	—		—	—	—	—	—			
A	356	12·09	10·11	0·421	0·562		1·99	1·56	0·162	0·270	2·08			
A	352	12·09	10·08	0·402	0·567		2·10	1·61	0·224	0·259	2·43			
1 R	320	10·33	8·50	0·331	0·611		2·01	1·54	0·123	0·345	2·18			
2 R	452	13·52	11·68	0·429	0·640		2·45	1·86	0·119	0·373	2·48			
3 R	370	12·45	10·52	0·360	0·509		2·13	1·67	0·122	0·366	2·39			
4 R	403	12·60	10·80	0·391	0·561		2·22	1·81	0·262	0·145	2·74			
5 R	370	13·35	11·40	0·402	0·570	1·169	2·32	1·81	0·137	0·371	2·54	1·302	0·385	1·537
6 R	417	13·10	11·40	0·409	0·582		2·07	1·67	0·133	0·273	2·54			
7 R	409	12·60	10·80	0·455	0·628		2·15	1·73	0·162	0·259	2·82			
8 R	409	13·86	11·80	0·471	0·621		2·38	1·94	0·147	0·299	2·70			
9 R	328	13·23	11·15	0·440	0·509		2·32	1·82	0·112	0·385	2·41			
10 R	385	13·48	11·70	0·441	0·555		2·28	1·89	0·158	0·234	2·66			
11 R	367	13·35	11·05	0·440	0·573		2·24	1·81	0·262	0·170	2·53			
12 R	428	13·23	11·28	0·462	0·572		2·29	1·90	0·137	0·259	2·52			
Intake		13·28					2·20				3·79		1·957	

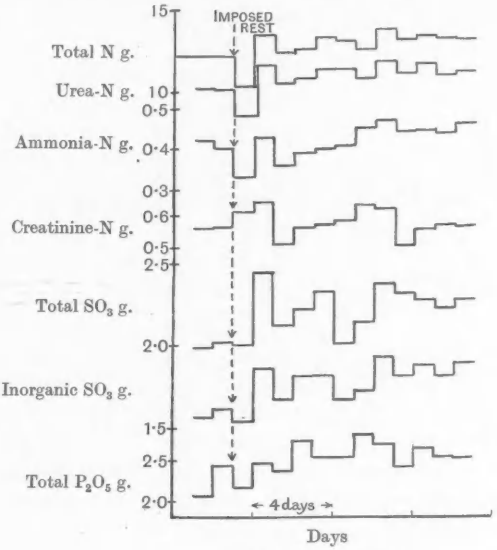

Fig. 3. Experiment 4.

Age 19 years; height 162 cm.; weight 50 kg. at start.

Daily intake of food:

Dried milk:	55 g.	Dried egg:	9 g.
Brown bread:	135 g.	Tea:	200 cc.
Digestive biscuits:	90 g.	Sugar:	15 g.
Fresh butter:	70 g.	Juice of 1 orange.	
Dried tomato soup:	50 g.	Marmalade:	1 level dessertspoonful.

Table V. (*Exp.* 5.)

State	Total nitrogen	Total sulphur (SO_3)	Total phosphorus P_2O_5
A	8·32	—	—
A	6·30	1·12	1·73
A	6·40	1·21	1·64
1 R	6·93	1·23	1·73
2 R	6·74	1·21	1·88
3 R	7·43	1·31	2·29
4 R	7·05	1·31	1·92
5 R	8·94	1·60	2·35
6 R	7·94	1·41	1·92
7 R	9·20	1·52	1·74
8 R	7·90	1·36	1·47

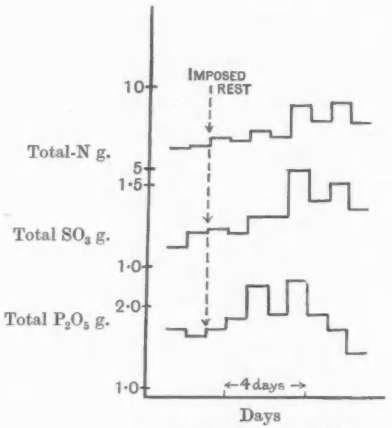

Fig. 4. Experiment 5.

Exp. 6. Subject, J.H., a miner in good physical condition, apart from occasional locking of one knee-joint, led a sedentary ward life for 3 days, prior to being confined to bed. No splint was used, and though the patient was not allowed up, he performed knee-bending exercises twice or thrice a day. Unfortunately, circumstances did not permit of a longer period of observation to decide what changes, if any, might ensue from confinement to bed without immobilisation. Following this experimental period, he was operated upon, being confined to bed for 10 days longer.

Age 20 years; height 165 cm.; weight 61·82 kg. at start.

Daily intake of food:

Dried milk:	55 g.	Dried apples:	15 g.
Brown bread:	200 g.	Tea:	400 cc.
Digestive biscuits:	90 g.	Sugar:	25 g.
Fresh butter:	40 g.	Juice of 1 orange.	
Steak:	200 g.	Marmalade:	1 teaspoonful.
Dried tomato soup:	15 g.		

Table VI. (*Exp.* 6.)

State	Volume of urine cc.	Total nitrogen g.
A	1220	14·00
A	1320	15·26
1 R+E	1750	13·58
2 R+E	1360	13·58
3 R+E	1150	12·18
4 R+E	1230	14·42
5 R+E	1340	13·72
6 R+E	1400	15·12
7 R+E	1290	14·00

A = active. R = resting. E = limited leg exercises.

Exp. 7. This female patient, M.G., a hawker, fractured her left humerus on 29. vi. 29. She led a sedentary life up till 15. vii. 29, when she was confined to bed, and was not allowed up for 32 days. Her urine was collected daily from the fifth day of rest till the eleventh day of resumed activity. The patient's movements in bed were in no way restricted. A menstrual period intervened in the course of the experiment. The resumed activity took the nature of sedentary ward life plus walking in the hospital precincts.

Age 37 years; height 156 cm.; weight 41·36 kg. without splint.

Table VII. (*Exp.* 7.)

State	Total urinary nitrogen. Daily average g.
5 R–11 R	8·75
12 R–17 R	8·65
Menstrual period.	
24 R–30 R	7·99
31 R–37 R	8·06
1 A– 5 A	8·40
6 A–11 A	8·80
Intake	9·28

Daily intake of food:

Dried milk:	55 g.	Steak:	100 g.
Brown bread:	175 g.	Tea:	400 cc.
Digestive biscuits:	90 g.	Sugar:	25 g.
Fresh butter:	60 g.	Juice of 1 orange.	
Dried tomato soup:	15 g.	Marmalade:	1 dessertspoonful.
Dried apples:	15 g.		

Exp. 8. The subject, M.A., in otherwise good health, suffered from a fractured tibia. He was kept on constant diet from the fifth day after fracture till his fourth day of resumed activity, a period of 48 days. His oxygen consumption was determined daily during this period (see Table X).

Age 20 years; height 180 cm.; weight 65·02 kg. on dismissal.

Table VIII.

		Partition of								
		Nitrogen					Sulphur SO_3			
Exp.	State	Total	Urea	Ammonia	Uric acid	Creatinine	Total	Inorganic	Ethereal	Neutral	State
I G.P.	A	12·45	—	—	0·35 (2·8)	0·63 (5·0)	2·08	1·58 (73·7)	0·17 (8·4)	0·37 (17·9)	A
	9 R-11 R	14·60	—	—	0·39 (2·1)	0·67 (4·5)	—	—	—	—	
							2·52	2·00 (79·2)	0·13 (5·4)	0·39 (15·4)	11 R
	4 A– 5 A	13·68	—	—	0·36 (2·6)	0·67 (4·9)	—	—	—	—	
II J.M.	A	11·93	9·72 (81·5)	0·43 (3·6)	—	0·54 (4·5)	1·94	1·41 (72·7)	0·19 (9·8)	0·34 (17·5)	A
	25 R	20·14	16·48 (81·8)	0·71 (3·5)	—	0·64 (3·1)	—	—	—	—	
							3·81	2·96 (77·7)	0·13 (3·4)	0·72 (18·9)	1 A
	3 A	17·36	15·25 (87·8)	1·05 (6·0)	—	0·59 (3·4)	—	—	—	—	
IV J.C.	A	12·09	10·11 (83·4)	0·42 (3·4)	—	0·56 (4·7)	2·05	1·59 (77·6)	0·19 (9·2)	0·27 (13·2)	A
	10 R–12 R	13·35	11·34 (84·9)	0·45 (3·3)	—	0·56 (4·2)	2·26	1·86 (82·3)	0·20 (8·9)	0·20 (8·9)	11 R

Figures in brackets are percentages.

Table IX.

Urinary ratios; sulphur : nitrogen.

Experiment	State	Days	Total output	Excess output	Retention	Excess output
I	A	1– 3	1 : 14·96	—	—	—
G.P.	R	1– 6	1 : 14·80	1 : 3·69	—	—
	R	7–11	1 : 14·98	1 : 15·16	—	—
	A	1– 5	1 : 13·74	1 : 6·87	—	—
II	A	1– 2	1 : 14·87	—	—	—
J.M.	R	1– 5	1 : 14·60	1 : 11·31	—	1 : 5·26
	R	6– 9	1 : 14·73	1 : 13·80	—	—
	A R	A– 7	1 : 13·57	1 : 8·50	—	—
	R A	8– 3 A	1 : 14·39	1 : 13·72	—	—
	A	4	—	1 : 14·08	1 : 13·78	—
III	A	1– 2	1 : 14·69	—	—	—
J.C.	R	1– 6	1 : 14·91	1 : 26·48	—	—
IV	A	1– 2	1 : 14·74	—	—	—
J.C.	R	1– 6	1 : 14·27	1 : 7·81	—	—
	R	7–12	1 : 14·59	1 : 13·25	—	—
V	A	2– 3	1 : 13·59	—	—	—
E.M.	R	1– 8	1 : 14·19	1 : 17·68	—	1 : 6·23

Nitrogen metabolism.

A practically constant excretion of urinary nitrogen was in existence in all cases prior to the super-imposition of the rest period. With the exception of Exp. 5, there was a drop in the excretion of urinary nitrogen on the first day of complete rest, and this was maintained sometimes for 1 or 2 days longer. The fact that there was no drop in this particular experiment may have been due to the patient's previous period of rest in bed. Following this initial diminution in nitrogen excretion, there is in all cases a distinct rise in

the urinary nitrogen, which is maintained irregularly. In Exp. 1, in the first days of imposed rest, there was a total excess output of 10·79 g. nitrogen in 11 days; in Exp. 2, 10·59 g. in 9 days; in Exp. 3, 2·33 g. in 6 days; in Exp. 4, 10·02 g. in 12 days; and in Exp. 5, 11·33 g. in 8 days.

Table X.

Gaseous metabolism.

Exp. 1. Subject: G.P. Oxygen consumption				Exp. 8. Subject: M.A. Oxygen consumption	
State	cc. D.B.	cc. Sp.	R.Q.	State	cc. Sp.
A	288	—	0·761	5 R–11 R	234 (+10 %, −12 %)
A	289	290	0·779		
A	286	281	0·763		
1 R	283	295	0·772	12 R–18 R	219 (+ 5·1 %, −11 %)
2 R	276	280	0·776		
3 R	270	279	0·755		
4 R	261	—	0·758	19 R–25 R	236 (+ 3·3 %, − 3·0 %)
5 R	253	257	0·804		
6 R	259	—	0·777		
7 R	242	242	0·818	26 R–32 R	235 (+ 3·4 %, − 5·3 %)
8 R	—	239	—		
9 R	255	264	0·818		
10 R	245	260	0·838	33 R–39 R	242 (+ 3·3 %, − 2·5 %)
11 R	244	241	0·840		
1 A	242	248	0·884		
2 A	249	250	0·845	40 R–49 R	240 (+ 2·1 %, − 0·9 %)
3 A	—	245	—		
4 A	258	265	0·785		
5 A	268	272	0·762	1 A– 4 A	244 (+ 1·6 %, − 0·8 %)

D.B. = Douglas bag method. Sp. = spirometric method.

In Exp. 2, during the days following the operation, there was a very marked rise in the output of urinary nitrogen, which, by the twenty-fifth day, had increased by almost 100 %. During this post-operation period, the patient's leg was not completely immobilised. Throughout the period, apart from a post-operative rise of 0·5°, there was no rise in body temperature. As it was not till the sixth day after operation that the nitrogen curve began to climb more steeply, it is probable that the effects of stovaine spinal anaesthesia and operative interference would have passed off. The possibility of some reflex trophic effect on muscle, accounting for this enormous rise in the excretion of nitrogen, cannot be discounted. It will be noticed also, that in this, as in some of the other experiments, the effect of resuming activity was not immediately noticeable, but that there was a lag period. The volume of urine passed during this latter period also rose, though the intake was unaltered, but was not of an order sufficient to suggest that the rise of nitrogen excreted might be the result of a washing out of the tissues; rather it suggested that the increased volume of urine was dependent on the increased excretion of solutes.

It can therefore be stated that muscular rest of the order described causes a practically immediate but small rise in the output of urinary nitrogen. As

the period of rest lengthens, it appears probable that the rate of increase is accelerated, but that eventually it declines to a more steady level, depending on the degree of immobilisation.

Distribution of nitrogen. The curve of the excretion of urea rises proportionately with that of total nitrogen. The excretion of ammonia, following the initial fall at the commencement of the rest period, takes longer to rise, but continues parallel to that of total nitrogen. It will be noticed that the total acidity also tends to rise (Exp. 4).

Spriggs [1907] found in primary muscular dystrophy that the diminution of creatinine in the urine was proportional to the muscular wasting. In the present series of experiments, the creatinine excretion was but little altered, a very slight rise sometimes being noted as the period of rest lengthened. Uric acid behaved similarly.

The excretion of faecal nitrogen and of total faecal solids remains practically constant (Exp. 1).

Metabolism of sulphur.

As early as 1881, Feder [1881] had noted that the sulphur of ingested protein was excreted earlier than the nitrogen. Rubner [1902], Hämäläinen and Helme [1907], and von Wendt [1907] in addition found that phosphorus followed the excretion of nitrogen. Wolf and Osterberg [1911] found a lag in the excretion of the sulphur of super-imposed egg-white, and Lewis [1916] found, on feeding starved dogs with meat, a lag in the sulphur output. Sherman and Hawk [1901], on the other hand, have shown an almost parallel excretion of sulphur and nitrogen. Cathcart and Burnett [1926] found that with moderately severe work the sulphur rose *pari passu* with the output of nitrogen. The accumulated evidence seems to indicate that it is the nutritive state of the organism at the time which determines the fate of the components of the ingested protein, but that normally there is a delay in the excretion of nitrogen [Wilson, 1925].

Just as the evidence based on the excretion of nitrogen indicates in these experiments an increased catabolism, so we find that the curves of the elimination of sulphur run closely parallel to that of total nitrogen, the sulphur, however, being excreted more rapidly than the nitrogen, as judged from the S : N ratios.

The rise in the output of sulphur is due to a slightly greater proportionate excretion of inorganic sulphates. There is a slight fall in the excretion of ethereal sulphates, the neutral sulphates remaining more constant.

Sulphur : Nitrogen ratios. The pre-rest values of all cases lay between 1 : 14·68 and 1 : 14·96, with the exception of Exp. 5, where the figure was 1 : 13·6. These ratios corresponded to the proportion of ingested sulphur and nitrogen which, in the majority of the experiments, was 1 : 14·9. In Exp. 5 the food intake was meat-free. With the exception of Exp. 3, the ratios of the total excess sulphur to nitrogen show the source of this loss to be

some sulphur-rich material. The ratio is highest during the early days of imposed rest. During the first 6 days of inactivity in Exp. 1 the excess output was 1 : 3·69. From the seventh to the eleventh days, it had fallen to 1 : 15·16. The other experiments provide additional evidence of a sulphur-rich source. The ratio of the excess output on the fourth day of resumed activity in Exp. 2 was 1 : 14·08, the ratio of the material which had stopped being excreted on that particular day being 1 : 13·78.

A possible explanation for the divergence of the S : N ratio in Exp. 3 may rest in the fact that the organism had only a very low reserve of sulphur, due to a previous period of immobilisation.

The generally accepted S : N ratio of muscle is 1 : 14. It is seen that non-use of the order described leads to a loss of sulphur and nitrogen, the source of the material being some sulphur-rich complex store material, which is probably intimately connected with muscle.

Metabolism of phosphorus.

As was indicated earlier in this paper, it was the excessive loss of phosphorus in fracture cases which drew the attention of the present writer to the question of the disturbed metabolism of inactivity. The rôle of phosphorus in metabolism has always presented great difficulties, and particularly the assessment of the relative sources of the excreted phosphorus. Benedict [1907] noted that in the case of starvation there was a tendency towards a much larger excretion of phosphorus in relation to nitrogen than occurs in the ordinary composition of flesh. The source of this extra phosphorus is generally believed to be bone.

The tables show that equilibration of phosphorus is much more difficult to attain than that of either nitrogen or sulphur. It was only on the day prior to the initiation of the rest periods in Exps. 2 and 4 that there was a definite correspondence between intake and output. In the previous note on the metabolism of sulphur, the known tendency of phosphorus to lag behind the excretion of both sulphur and nitrogen was discussed. Another factor which may have interfered in part with early equilibrium was the relative richness of the diets in phosphorus and calcium.

The curves of the urinary excretion of phosphorus run, with varying regularity, parallel to those of nitrogen. The excretion of faecal phosphorus (Exp. 1) is little altered. As the period lengthens, there is a slight rise, corresponding to the rise in calcium excretion.

The usually accepted ratio of P_2O_5 : N in muscle is 1 : 6·6. Assuming the intake of P_2O_5 as the basal figure, then the excess output of P_2O_5 during the first 7 days of rest in Exp. 2 is 1·16 g. The excess output of nitrogen during the same period is 6·64 g., using the urinary basal figure of 11·9 g. daily. The P_2O_5 : N ratio is 1 : 5·7. In Exp. 4, using similar methods, the ratio is very low, 1 : 14·3. In Exp. 5, the ratio of the urinary excess outputs is 1 : 6·23.

The data provided by these experiments are too inconclusive to allow us

to form a definite conception of the rôle of phosphorus in the metabolism of imposed rest. Longer periods of immobilisation are required before a clear result can be obtained.

Metabolism of calcium.

Calcium equilibrium takes longer to attain than phosphorus. It was not attained in either Exps. 1, 2 and 4 until the period of inactivity was well advanced. There appeared to be a retention up to that point. Whether this was due to the high calcium content of the diet, or not, cannot be definitely stated. There appears to be a tendency for a loss of calcium to occur as the period of rest lengthens. The experimental data here do not warrant any more definite statement.

The experiments of Allison and Brooks [1921, 1922] have demonstrated by non-chemical methods that bone atrophy through disuse can readily take place.

It would appear essential that all experiments dealing with the metabolism of phosphorus and calcium must be of much longer duration and have much longer pre-periods than those dealing with the metabolism of nitrogen and sulphur.

Gaseous metabolism.

The work of Hanriot and Richet [1888], Lehmann and Zuntz [1893], Benedict [1907], Takahira [1925] and Wishart [1927] has shown that during fasting the metabolism is very constant from day to day, even when the respiratory quotient is falling, but that essentially there is a reduction of metabolism. The data obtained in the course of the present series of experiments point to a similar constancy during prolonged muscular rest in the adequately fed subject. The diet of both subjects was kept constant from day to day, and all gaseous determinations were performed in the morning, with the subjects in the post-absorptive state, having lain quietly in bed for at least 12 hours. The subjects were well practised previously. In Exp. 1 determinations of the oxygen consumption were obtained both by the collection of expired air in a Douglas bag, with subsequent analysis in a Haldane's gas analyser, and with a calibrated spirometer of the Benedict-Collins type. The slightly greater facility of respiration in an open circuit compared with a closed one would account for the fact that the spirometric record is usually a little higher than that of the Douglas bag method. The maxima and minima percentage variations from the mean in the various periods of observations of subject M.A. during an experimental period of 48 days (Exp. 8) decreased progressively as the period lengthened, until they became practically negligible. From $\pm$ 11 % they dropped to $\pm$ 1·2 %. Observations on a patient whose oxygen consumption was determined over a period of 40 days revealed, again, a gradual diminution from $\pm$ 14 % to $\pm$ 1·5 % in the maxima and minima percentage deviations from the mean of 6-day periods (data regarding

this patient are held over for a subsequent communication on the effect of massage on metabolism).

Since the level of activity is constant from day to day this gradual reduction in oscillation must be due to increased custom diminishing mental and physical disturbance. This point raises the question as to whether the latitude of $\pm$ 15 % day to day variation generally allowed in normals is due to insufficient practice.

Although in the subject G.P. there tended to be a slight but steady diminution in the oxygen consumption, which was accompanied by a rise in the respiratory quotient as the period of rest lengthened, this diminution did not fall below 17 % from the pre-rest values during the period of observation. A tendency for a fall in the oxygen consumption of subject M.A. was also noted, but thereafter the metabolism maintained a very constant level. In this connection, the work of Harding [1926] is interesting. She noted that the oxygen consumption of muscles wasted as the result of arthritis is definitely increased above normal, whereas that of muscles wasted from disuse is unaltered.

Discussion.

In the present series of experiments, it has been demonstrated that prolonged rest in healthy subjects leads to a loss of nitrogen, sulphur, phosphorus, and in less degree, calcium. These changes must be reckoned as due to non-use of the body generally, and most probably to non-use of muscle and bone in particular.

Cathcart and Burnett [1926] and others have shown that moderately severe work produced a definite, though small, rise in the output of nitrogen and sulphur, signifying that the increased catabolism was in excess of any definite stimulation of anabolism. It is very curious that when the experimental conditions are changed to the other extreme, the urinary picture remains strikingly similar. That such a loss does not continue indefinitely during moderately severe periods of muscular work has been shown by Wilson. He has communicated to the present writer the results of a personal experiment, where a much longer period of work was performed, resulting in a fall of the curves to, and even below, the basal line. Surveying the results obtained in work-experiments with those of the present series on rest, it would appear that when one alters the level of functional activity there is a lag period before nitrogen equilibrium is again obtained. During this period there is a loss of the essentially active substance, which persists until, in the case of increased muscle work, the anabolic response is equal and opposite to, or even greater than, the increased catabolism. In the case of decreased muscular activity, it persists until the amount of functioning metabolic substance is reduced to such a level that the catabolism is equal and opposite to the diminished anabolism. Such a change does not appear to alter appreciably the endogenous metabolism as represented by uric acid and creatinine.

The reason for the absence of any immediate diminution of the urinary nitrogen and sulphur excretions on the resumption of activity in these experiments is probably that the anabolic response is masked by a sweeping-out of the catabolic products from the relatively stagnant tissues.

It appears reasonable to assume that the term "autolysis" may be applied to these processes in muscle. According to Morse [1914] disused muscle appears not to have any increased capacity for autolysis. Bradley [1918, 1922], however, has shown that voluntary muscle autolyses slowly and incompletely, and is influenced by the tissue reaction. He considers that, as a result of inadequate blood supply, the catabolic products accumulate, resulting in a rise in the $H^{\cdot}$ ions within the cell, and a change from basic to acidic proteins. This activates the proteolytic enzymes, particularly the primary protease. The ereptase, previously inoperative, has now a suitable substrate, and the muscle mass decreases until equilibrium between the cell and its blood and lymph supply is re-established.

Such a hypothesis appears to offer a convenient description of the phenomena observed, but is it certain that the diminished blood supply is the cause and not yet another effect of the decreased metabolism? Any ultimate conception must interpret all these effects as being due to a primary biological necessity.

Summary.

1. Subjects in nitrogenous equilibrium show, within a day or two from the commencement of a period of muscle rest of the order described, a rise in the excretion of sulphur, nitrogen, phosphorus, and calcium, in that order of priority. This loss is maintained fairly steadily for a varying period, after which it gradually declines.

2. The rise in the excretion of sulphur is due to a practically proportionate increase in inorganic sulphate. Ethereal sulphate tends to decrease, while neutral sulphur remains more constant.

3. The rise in the excretion of nitrogen is mainly due to a proportionate increase in the amount of urea. Ammonia excretion also rises, but more slowly. Creatinine and uric acid are practically unaltered.

4. The S : N ratio suggests a sulphur-rich source of the excreted material, presumably for the most part muscle.

5. Apart from a slight fall in the oxygen consumption, associated with a rise in the respiratory quotient, the gaseous metabolism remains very constant from day to day.

6. Maxima and minima percentage day to day variations in the oxygen consumption gradually decrease as the experimental period lengthens, from $\pm 14\,\%$ to $\pm 1{\cdot}2\,\%$.

In conclusion I desire to thank Prof. E. P. Cathcart for his very helpful criticism. I also wish to express my thanks to Prof. P. Paterson and Messrs Milne MacIntyre, Patrick and Taylor for their active cooperation in supplying me with suitable subjects.

I am grateful to Dr James MacFarlane for a grant in aid of this research.

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CXLVI. THE EXTRACTIVE PURINES OF MUSCLE. I.

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(*Received October 14th, 1929.*)

Determinations of the purine-nitrogen content of dog and rabbit skeletal muscle before and after extraction with boiling water show that the proportion of extractive purines is very considerable, amounting to over 50 % of the total muscle-purine content. The extractive purine content is fairly constant, and appears to be independent of the state of nutrition [Dmochowski, 1928, 1, 2].

In view of the fact that purine-nitrogen content has been taken as a base for researches on reserve protein, and also in view of the conflicting results obtained by previous workers in this field, it appeared necessary once again to investigate both quantitatively and qualitatively the purine substances present in skeletal muscle.

A review of the literature dealing with muscle-purines, in particular with extractive purines, shows a state of chaos. We have in another paper [Dmochowski, 1929] given a critical review of these papers; the discrepancies between the results of the various authors are illustrated by Table I, from which it will be seen that the purine-nitrogen content of muscle extracts as given by different authors varies from 2 to 129 mg. per 100 g.

Table I. *Purine-N content of whole muscle and of aqueous extracts in mg. per 100 g.*

Author	Whole muscle	Aqueous extract	Animal
Demant [1879]	—	(3–6)*	Starved pigeon
Kossel [1882, 1884]	(30–60)	—	Ox, dog, chicken
Salomon [1881]	—	(30)	Dog
Schittenhelm [1905, 1910]	—	40–50	Pig, ox
Burian and Hall [1903]	55–72	—	Ox, horse
Buglia and Costantino [1912]	68	—	Ox
Scaffidi [1910]	35	3	Frog
Scaffidi [1911]	61	9	Ox
Yoshimura [1911]	—	(2)	Wild rabbit
Suzuki and Yoshimura [1909]	—	(12)	Herring
Fürth and Schwartz [1910]	—	23–36	Dog, horse
Smorodincev [1913, 1914, 1922]	—	(3–50)	Horse, ox, sheep, pig
Offer and Rosenqvist [1899]	—	25–50	Ox, calf
Adler [1908]	—	14–129	Ox, calf
Bessau and Schmidt [1910]	—	15	Ox
v. Fellenberg [1918]	(50–55)	(28)	Ox, calf, frog
Kikuchi [1923]	58–83	—	Rabbit
Truszkowski [1927]	54–80	—	Dog, horse, calf, rat
Dmochowski [1928, 1, 2]	80–104	—	Dog, rabbit

* Values in brackets have been calculated by me as purine-N from the total purine content.

These discrepancies are, as will be shown in this paper, due to the presence in muscle extracts of purine-containing complexes.

Experimental.

Aqueous extracts from dog and rabbit skeletal muscle were taken as experimental material. The animals were killed by intracardial injection of chloroform, immediately after which muscle tissue from the hind limbs, as well as in some cases dorsal muscle tissue, was removed, passed with the least possible delay through a mincing machine, and 100 g. portions were thrown into 500–1000 cc. of boiling water. The mixture was boiled for 10–15 minutes, after which it was filtered, and the residue extracted a further two or three times. The united filtrates were feebly acidified with acetic acid, in order to coagulate proteins, and the protein-free filtrate was concentrated to about 400 cc. Its total nitrogen content was determined by the method of Kjeldahl, and the purine-N content before and after 4 hours' hydrolysis with 4 % sulphuric acid by the method of Krüger and Schittenhelm [1905]. The unhydrolysed extract contained on the average 5 mg. purine-N per 100 g. tissue, whilst after hydrolysis 30–50 mg. per 100 g. tissue were found.

Table II.

	Purine-N (as mg. per 100 g. tissue) content of				
	Extract				
Total N content %	Unhydrolysed	Hydrolysed	Whole muscle	Residue after extraction	Remarks
0·56	8·7?	20·0?	109·75	39·07	Rabbit, fed
0·47	5·0	42·3	87·35	43·78	,, ,,
0·44	6·7	—	—	—	,, ,,
—	3·8	44·3	—	30·6	,, ,,
—	3·4	44·2	85·8	—	,, ,,
—	—	33·9	123·3?	74·19?	,, fasting
—	5·0	—	95·5	46·46	,, ,,
0·48	—	45·3	91·97	39·28	,, ,,
0·65	5·1	—	101·5	47·22	,, ,,
—	—	53·0	99·3	44·5	,, fed
0·52	4·8	43·8	95·9	41·6	—
0·40	8·8	31·36	79·37	32·32	Dog, fed on meal
0·35	5·7	34·3	79·13	30·61	,, fed on meat
—	5·6	27·5	—	—	,, ill

The results, given in Table II, show that over 50 % of the purine-N of muscle tissue is found in the hydrolysed extract, whilst the remainder cannot be removed even by prolonged extraction. Free purines are present in very small quantity—about 5 % (of the total purine-N) in whole muscle, or 10 % for the extract alone. Slightly larger quantities are found in material which is not absolutely fresh.

With the object of investigating the influence of hydrolysis on the quantity of purines precipitated, or of the inhibitive influence of the colloids present on the precipitation of the various purine compounds, hypoxanthine, which is most probably the free purine present in muscle, or nucleotide (crystalline adenylic acid, prepared from Boehringer's sodium yeast nucleinate by the

method of Steudel and Peiser [1924]), was added to aqueous dog muscle extract, after which purine-N was determined by the method of Krüger and Schmidt.

It was found that hypoxanthine was in these circumstances practically quantitatively precipitated, adenylic acid hardly at all. Thus 27·6 mg. purine-N was found in an extract originally containing 16·2 mg. and to which a quantity of hypoxanthine equivalent to 10·4 mg. purine-N had been added, whilst in a different case the apparent purine-N content remained unchanged in spite of the addition of an amount of adenylic acid corresponding to 52·9 mg. purine-N.

Since adenylic acid is, as was previously shown by Thannhauser and Czoniczer [1920], readily precipitable from aqueous solution, and since this is not the case with aqueous muscle extracts, it would appear that here lies the cause of the low results obtained by many authors for extractive purines, as well as of the higher values obtained after hydrolysis.

Should it actually be the case that the nucleotides, adenylic and inosinic acids, are present in the aqueous extract, as would appear from a consideration of the solubilities of these acids and their salts to be extremely probable, then it might be expected that after the precipitation of these nucleotides with uranyl acetate according to Thannhauser and Czoniczer, much lower values for purine-N would be obtained for the hydrolysed filtrate.

This supposition was confirmed experimentally on rabbit muscle extract, which was found after hydrolysis to have a total content of 44·3 mg. of purine-N, whilst after precipitation with 1·5 % uranyl acetate and hydrolysis of the filtrate the content was only 12·4 mg. The residue, which contained 28 mg. purine-N, gave, after removal of uranium, the pentose reaction in its purine fraction. This reaction was negative in the case of the "free" purines precipitated from the unhydrolysed extract as copper compounds, as were also the murexide and Kossel's reactions. A positive Weidel reaction and a very feeble xanthine reaction were given in the latter case.

Having to a certain extent determined the proportions of purine-N in the various fractions of extractive purines, *i.e.* nucleotide, nucleoside and "free," as well as of those remaining after extraction, it remained to determine quantitatively the constituent purines, guanine, adenine, hypoxanthine and xanthine in the separate fractions.

Up to the present, aqueous extracts of rabbit and chicken muscle (300–1600 g.) have been analysed, as well as the residue after extraction [Dmochowski, 1929]. The nucleotide was separated from the nucleoside fraction and each was hydrolysed and analysed separately with certain modifications by the method of Steudel [1924] (double or triple precipitation as cuprous salt and single precipitation as silver salt).

The results, given in Table III, indicate that very small quantities of pure guanine are present, and this exclusively in the residue after extraction. Xanthine is found only in traces.

Table III. *The individual purines of rabbit and chicken muscle tissues and their content as mg. purine-N per 100 g. tissue.*

Material	No. of animal	Quantity g.	Guanine	Guanine-N	Adenine picrate	Adenine-N	Hypoxanthine-N
Extracted rabbit muscle	I	300	10·0	4·6	13·4	2·6	>0·7
	II	1000	5·8	2·7	—	—	—
	III	950	>4·63	>2·13	16·38	3·24	1·0
	IV	950	8·0	3·7	16·5	3·3	—
	V	500	10·0	4·63	23·6	4·7	—
Total extract from same	I	300	0	0	103·5	19·9	>2·16
Uranyl acetate precipitate from extract of same	III	950	0	0	87·0	17·2	—
	IV	950	0	0	78·8	15·7	4·3
Filtrate from same	III	950	0	0	16·1	3·20	5·8
	IV	950	0	0	0	0	>2·9
Extracted chicken muscle	V	1600	7·9	3·6	41·6	8·2	2·0
Uranyl acetate precipitate from extract of same	V	800	0	0	38·5	7·6	10·26
Filtrate from same	V	800	0	0	9·1	1·8	>5·8

Adenine picrate, M.P. 278–280°, was precipitated in a fairly pure, crystalline state from the extracts, whilst that from the residue crystallised only after reprecipitation as silver salt. Hypoxanthine was at first determined as argentipicrate [Steude, 1924], but as the crystallisation of hypoxanthine picrate does not take place quantitatively, and as the supposed silver precipitate of xanthine turned out to be in reality one of hypoxanthine, giving the characteristic nitrate, the quantity of hypoxanthine-N was calculated directly from the weight of the corresponding silver precipitate according to the formula $C_5H_2ON_4Ag_2.H_2O$.

Discussion.

An examination of four different forms in which purines occur in muscle tissue shows that the proportion of free purines, extractible by hot or warm water and directly precipitable from the extract as copper compounds, is extremely small. In the case of rabbit muscle, containing on the average 80–100 mg. purine-N per 100 g., the mean free purine-N content is 5 mg. per 100 g. Nor is the value markedly increased by leaving the comminuted muscle for short periods of time, or by hydrolysing the extract during 2–3 minutes. Guanine, adenine, and uric acid (negative murexide reaction) are absent from this fraction.

The hydrolysis of the aqueous extract, using 4 % sulphuric acid, leads to a considerable increase in the amount of purines precipitated by copper sulphate in the presence of sodium hydrosulphite. The purine-N content now becomes 40 mg. per 100 g. for rabbit and 30 mg. per 100 g. for dog muscle extracts, an amount equivalent on the average to 50 % of the total quantity of purines present in muscle. This observation explains the cause of the low values obtained for extractive purines by many authors, such as Scaffidi [1910], who gives the purine-N content of frog muscle as 35 mg. per 100 g.,

and that of the extract as 3 mg., whilst our determinations for fresh muscles of *Rana esculenta* show that the respective figures are 54 and 23 mg. per 100 g. Similarly, the small quantities of purines precipitated by phosphotungstic acid from muscle extracts by Smorodincev [1913, 1914, 1922] may be ascribed to this cause.

The supposition that plant nucleic acid, which is similarly not precipitable under our conditions, occurs in muscle tissue extracts had to be abandoned in view of the absence of effect of gentle alkaline hydrolysis, as also of the absence of guanine from this fraction. The non-precipitability of adenylic acid added to muscle extract leads to the conclusion that considerable quantities of nucleotides, precipitable from aqueous solution but not from the extract, are present in muscle tissue.

The addition of uranyl acetate to the extracts leads to the precipitation of pentose nucleotides, which constitute about 75 % of extractive purines. The filtrate from the uranyl acetate precipitate contained free and combined purines, the latter probably as nucleosides.

The values found for the purine-N contents of the nucleotide and nucleoside fractions agree fairly well with those of other authors. Thus Gudzent and Keeser [1922] found in human and chicken muscle 3–7 mg. of non-nucleotide purine-N per 100 g. tissue and a large quantity, not determined, of nucleotides.

The highest value given by Parnas [1928] for loss in adenine of rabbit muscle after trituration amounts to 32 mg. purine-N per 100 g. tissue, a value agreeing fairly well with those obtained in this research.

Grund's [1902] value for the pentose content of ox muscle is somewhat low (23 mg. per 100 g., corresponding to only 11 mg. of nucleotide- and nucleoside-N); it has, however, been shown by Schmidt [1928] that the pentoses of adenylic acid of muscles readily escape detection.

Apart from the early work of Kossel [1884] no accurate figures for the individual purines of muscles are available.

Our preliminary researches show that guanine is absent from the extract, and is present with an equal amount of adenine only in small quantity (6–10 mg. per 100 g.) in the residue after extraction, probably representing exclusively nuclear nucleic acid. By far the greater part (20 mg. adenine-N per 100 g.) of the adenine present in rabbit muscle is to be found in the extract, mostly as adenylic acid (17 mg. adenine-N), whilst the hypoxanthine of fresh muscle is present preponderatingly in the free state.

Under the experimental conditions applied in this research (immediate immersion of comminuted muscle in boiling water) deamination of adenylic acid does not take place, as is shown by the figures obtained for inosinic acid hypoxanthine. The small quantity of adenine remaining sometimes in the extract after precipitation of nucleotides may possibly be due to adenosine. The traces of hypoxanthine found in the extracted residue are probably due to incomplete extraction.

An important fact emerging from our researches is that the sum of

guanine-, adenine- and hypoxanthine-N found (xanthine was not found) corresponds to only two-thirds of the total purine-N content of muscle. A purine fraction was isolated, giving a soluble picrate, but not giving Weidel's or the xanthine reaction, or the characteristic nitrate of xanthine. A part of these purines is, in the case of rabbit muscle, precipitable by ammonia as a gelatinous mass from which a crystalline sulphate can be prepared. This sulphate is not identical with that of hydroxyadenine, since it possesses water of crystallisation. Research on this new fraction is now being prosecuted.

The above research was, with the exception of that part of it dealing with the individual purines, commenced in the spring of 1927; the results obtained were communicated to the Polish Biological Society on June 20th, 1928, and a summary of this paper has been published [Dmochowski, 1928, 1]. Parnas's [1928, 1929] important papers and that of Embden and Wassermeyer [1928] were published at a later date, the results of these authors being partly concordant with ours.

Parnas relegates nearly the whole purine-N content of frog muscle of about 30 mg. per 100 g. tissue to nucleotides, whilst Embden and Wassermeyer [1928] find considerably larger quantities of ammoniagenic nucleotide-N precipitated by copper sulphate and calcium hydroxide, viz. 40–80 mg. purine-N per 100 g. tissue for frog muscle. The values obtained by this author, as compared with those of Parnas and of Dmochowski, would indicate that adenylic acid and adenine are not the only bodies present in muscle capable of giving rise to ammonia; possibly the unidentified purine substances found by us are the source of this extra ammonia.

Summary.

1. It has been shown that about 50 % of the purines present in muscle are to be found in the aqueous extract, which, however, contains only traces of "free" purines, precipitable as cuprous salts from the unhydrolysed solution (5 mg. purine-N per 100 g. tissue for dog and rabbit muscle).

2. By far the greater part of extractive purines (40 mg. purine-N per 100 g. tissue) are precipitable only after hydrolysis of the aqueous extract with 4 % sulphuric acid, and 75 % of this fraction is contained in pentose nucleotides, precipitated by uranyl acetate; the filtrate contains, apart from "free" purines, probably also nucleosides.

3. Hunger and different conditions of alimentation do not appear to affect the distribution of the various purine fractions.

4. Quantitative determinations of the individual purines indicate the absence of guanine from rabbit muscle extracts, and the presence in the residues of 2·7–4·6 mg. of guanine-N per 100 g. tissue, with the same adenine-N content. These purines probably therefore originate from nuclear nucleic acids. Most of the adenine present in fresh muscle occurs in the nucleotide fraction of extracts, whilst the hypoxanthine is found in the nucleoside fraction, probably as the free purine. Xanthine is found only in traces.

5. A considerable purine fraction giving a soluble picrate was isolated both from the extracts and residues of rabbit muscle. The purines of this fraction are precipitable by ammonia and yield a crystalline sulphate different from those of guanine and of hydroxyadenine.

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CXLVII. THE BIOLOGICAL DECOMPOSITION OF PLANT MATERIALS.

I. THE NATURE AND QUANTITY OF THE FURFURALDEHYDE-YIELDING SUBSTANCES IN STRAWS.

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(*Received October 21st, 1929.*)

Introductory.

The biological decomposition of cellulosic materials is a process which is obviously bound up with the chemical composition of the plant tissues, and the availability of the constituents to the members of the micro-flora present. Rege [1927] states that two factors appear to control the decomposition of cellulosic material in the presence of sufficient assimilable nitrogen. The first is the food or "energy" factor, which he associates with the "pentosans," and the second, the "inhibitory" factor, which is the lignin fraction. He claims that it is possible to predict the decomposability of any material, for if the ratio of "energy" factor to "inhibitory" factor, that is "pentosans" to lignin, is more than unity, the material is easily decomposed, but if it is below 0·5 the material is very resistant to microbial attack. Work on somewhat similar lines has been carried out by Waksman and Tenney [1926, 1927, 1, 2, 1928]. They recognised that the most resistant constituent of plant tissues is the lignin fraction, which accumulates during the process of rotting, whilst the soluble materials, the cellulose and the pentosans, are progressively removed.

In the investigations to be described in a further communication, the point at issue was the relative importance of the various furfuraldehyde-yielding substances and cellulose. For this purpose a method of differentiating these substances had to be devised. Both Rege [1927] and Waksman and Tenney [1926] obtained a figure for "pentosan" by employing the Kröber factor on the total furfuraldehyde yield. Figures obtained in this way have little or no significance, since they bear no relationship to the actual quantity of furfuraldehyde-yielding constituents of the particular material under investigation. In point of fact, low results are invariably obtained, since the use of this factor involves the assumption that the furfuraldehyde-yielding substances present consist solely of pentose units, an assumption which cannot possibly be defended in view of the large amount of experimental evidence to the contrary. Furfuraldehyde may be obtained from two groups on

distillation with mineral acids—the pentose unit yielding about 75 % of the theoretical amount, and the uronic acids, giving 33 % of their weight. These groups both occur together with hexose units in various proportions in the hemicelluloses, gums, and pectin. The presence of hexose units obviously lowers the furfuraldehyde yield of the product below that which the Kröber factor would indicate. For example, 20 parts of furfuraldehyde might be derived from about 32 parts of xylan, from 100 parts of pectin, or from 61 parts of the hemicellulose A from rye straw, and the use of the Kröber factor, which in this case would indicate 35 % of "pentosan" is manifestly meaningless. In the work the source of the furfuraldehyde has in each case been investigated, so that the figures for the furfuraldehyde-yielding constituents may be obtained as a close approximation to the quantity actually present.

Methods of analysis.

The material for analysis was evenly but not too finely ground, and the following analyses carried out.

Total furfuraldehyde yield. The standard method of boiling with 12 % hydrochloric acid was followed, the furfuraldehyde being precipitated with phloroglucinol. The phloroglucide was extracted with boiling alcohol to remove traces of the phloroglucides of methylfurfuraldehyde and ω-hydroxymethylfurfuraldehyde.

Carbon dioxide yield. The modification of Lefevre and Tollens's [1907] method as proposed by Nanji, Paton and Ling [1925] for the estimation of uronic acids was employed.

Calcium pectate yield. Since it is not possible to determine the actual form in which pectin is present in the straw it is conveniently expressed as calcium pectate, being estimated by extraction with 0·5 % ammonium oxalate according to the method of Nanji and Norman [1928].

Cellulose. Despite certain objections which will be discussed later, the chlorination method due to Cross and Bevan [1918] was employed with certain modifications. The chlorination was actually carried out on a small Büchner funnel, so that a stream of the gas could be drawn through the straw and proper penetration effected. The preliminary treatment for 20 minutes with 100 cc. of 1 % NaOH as originally proposed by Cross and Bevan was employed, even though this has been adversely criticised by several workers, notably Renker [1910], on the grounds that the cellulose yield is lowered thereby. It does, however, serve to remove a portion of the encrusting hemicelluloses, so that the chlorine may have full access to the lignin, and in this way, and also, because it ensures complete wetting, lowers the time necessary for chlorination. Over-chlorination is to be avoided owing to oxidation, and the long periods used by some workers are unnecessary if care is taken to ensure proper penetration. For the straw used in this work, three periods of 12, 7 and 5 minutes were found to be sufficient to free the cellulose from

lignin. The final washing must be very thoroughly carried out with hot water, since, if washing is unsatisfactory, charring takes place on drying, owing to the traces of sulphuric acid formed from the permanganate and sulphurous acid, after the bleaching process.

The product obtained by the chlorination method cannot be regarded as consisting solely of pure cellulose. The principle of the estimation is the resistance of certain of the cell wall substances to attack by chlorine and sulphite, and in this the mechanical structure of the tissue is not unimportant. It is found that the "cellulose" isolated by the Cross and Bevan method from plant tissues almost invariably yields a considerable quantity of furfuraldehyde, due to furfuraldehyde-yielding substances intimately associated with the cellulose. Many workers correct the percentage yield of cellulose for "pentosan" using the Kröber factor, thus obtaining a somewhat truer estimate of the real cellulose content. This adjustment has been criticised on the grounds that the furfuraldehyde may be in part derived not from pentosans but from oxidation products formed from cellulose during the chlorination. This objection will be discussed later.

Lignin. The usual methods for the estimation of this constituent depend on the fact that it is chemically very resistant, and that by treatment with strong acid all other substances are destroyed, leaving the lignin alone. It is certain however that the lignin is changed in some way by this treatment since the final product differs in methyl and acetyl content and is insoluble in sulphite. A more direct method, by extraction with alkali under pressure, has been proposed by Mehta [1925], but this, though theoretically sound, appears to remove only a certain portion of the lignin, the results being considerably lower than by the acid methods. Willstätter and Zeichmeister [1913] proposed the use of hydrochloric acid of *d.* 1·21, but this has the disadvantage that, unless ice is used for cooling, the strength of the acid diminishes and the results are high. The method due to Ost and Wilkening [1910], involving the use of 72 % sulphuric acid, gives results which are more consistent; and a modification of it put forward by Schwalbe [1925] was employed in this work. 2 g. of the dried material are treated with 60 cc. of 72 % H_2SO_4 and 15 cc. of 18 % HCl for 24 hours, the whole being kept in a cold water-bath. At the end of this period, about 500 cc. of distilled water are added and the whole is boiled for half an hour. This final treatment completes the hydrolysis leaving a product which filters readily on a Gooch crucible through paper. After thorough washing it is dried, weighed and ashed, the weight of lignin being corrected for the ash present, which is usually small.

Experimental.

A complete examination of the chief constituents of the two straws used in this investigation, namely oat straw and rye straw, was first made; and it will be convenient now to discuss the former, group by group.

The analytical figures obtained were as follows.

Oat straw.

	%
Ash	7·07
Total furfuraldehyde yield	16·08
Carbon dioxide yield	1·21
Calcium pectate yield	1·10
Cellulose (Cross and Bevan)	53·14
Furfuraldehyde from cellulose	5·99
Lignin	18·54
Protein	1·8

Determination of hemicellulose content.

It is well known that the hemicelluloses contain considerable quantities of uronic acids and, as mentioned above, these acids yield a certain amount of furfuraldehyde. The total furfuraldehyde may be derived, therefore, from the pentose and uronic groups in the hemicelluloses, from the pectin, and from the rather obscure substances in the Cross and Bevan cellulose fraction. It is possible from the data above to disentangle these various sources, and to present an ordered balance sheet of the constituents.

Let A represent total furfuraldehyde yield.

„ B „ furfuraldehyde from cellulose fraction.

„ C „ furfuraldehyde from pectin (19·5 % on calcium pectate).

„ D „ furfuraldehyde from total uronic acid anhydride (16·66 %).

„ E „ furfuraldehyde from uronic acid anhydride in pectin (11·6 % on calcium pectate).

From this (C–E) represents furfuraldehyde from the arabinose group in pectin. Then the figure for furfuraldehyde arising from the pentose groups of the hemicelluloses present in 100 g. straw is given by

$$[A - \{B + D + (C - E)\}]$$

which in the case of this straw is

$$[16{\cdot}08 - \{5{\cdot}99 + 0{\cdot}81 + (0{\cdot}21 - 0{\cdot}13)\}] = [16{\cdot}08 - 6{\cdot}88] = 9{\cdot}2\ \%.$$

In order that this might be interpreted in terms of hemicellulose it was necessary next to determine the actual furfuraldehyde yield, since, as mentioned above, it is not possible to assume that only pentose groups are present. The method of extraction was suggested by Norris and Preece [private communication]. 100 g. of the finely powdered straw were extracted on a boiling water-bath with 1 litre 0·5 % ammonium oxalate twice for a period of 4 hours each to remove the pectic substances, after which it was well washed with boiling water. This was followed by two treatments for 2 hours each with a litre of 55 % alcohol containing 1 % NaOH, with the object of extracting the more readily soluble lignin. After washing by a short treatment with 55 % alcohol alone, the extraction of hemicelluloses was effected by four treatments with 4 % NaOH, the first for 4 hours, and the others for 2 hours

each. The combined alkaline extracts of the hemicelluloses were filtered through packed glass wool, and acidified with glacial acetic acid. A colloidal solution was obtained, from which the mixed hemicellulose product was precipitated by addition of alcohol. After filtering, the precipitate was washed with acetic acid and alcohol mixture, and redissolved in dilute NaOH. Re-precipitation was carried out as before by acidification with acetic acid and addition of alcohol. The product was dried with alcohol and ether.

The yields of furfuraldehyde and CO_2 were determined on this mixed product.

	%
Furfuraldehyde yield on ash-free basis ...	43·0
CO_2 yield on ash-free basis	3·9
Uronic acid anhydride	15·6
Furfuraldehyde due to uronic groups ...	2·6

By difference, the furfuraldehyde due to pentose units of hemicellulose = 40·4 %. Since it was determined above that the amount of furfuraldehyde arising from the pentose groups of the hemicelluloses present in 100 g. straw was 9·20, and the pentose units in the hemicelluloses themselves yield 40·4 % furfuraldehyde, it is clear that an approximation for the actual hemicellulose content is given by the quotient, namely 22·8 %.

The nature of the hemicelluloses present.

The nature of the hemicelluloses present in this mixture was next investigated. The mixed product was separated into two portions, hemicellulose A and hemicellulose B according to the method proposed by O'Dwyer [1926]. There is reason to suppose that these also are not individual entities but mixtures. The mixed product was dissolved in NaOH and precipitated by large quantities of glacial acetic acid. The excess of acid results in the hemicellulose A fraction being precipitated in an easily filterable form. To the filtrate was added alcohol thereby precipitating hemicellulose B. Purification was carried out in each case by repetition of the same procedure.

Oat straw.

	Hemicellulose A %	Hemicellulose B %
Furfuraldehyde yield on ash-free basis ...	42·9	43·3
CO_2 yield on ash-free basis	2·7	7·95
Uronic acid anhydride	10·8	31·80
Furfuraldehyde due to uronic groups ...	0·65	1·90
Furfuraldehyde due to pentose groups...	42·25	41·5

The amount of the former obtained was much in excess of the latter, indeed from the uronic content of the mixture it would seem that the proportion lies between three and four to one.

Hydrolysis of each was carried out with 2 % H_2SO_4 for 6 hours at 100°,

the liquid being then neutralised with barium carbonate and the filtrate concentrated under reduced pressure to a thin syrup.

The sugars tested for were mannose as hydrazone, arabinose as the diphenylosazone, xylose as the cadmium xylonobromide salt, and galactose as mucic acid. It was not possible in either case to characterise the uronic acid present owing to the small quantity available.

Preparation	Mannose	Arabinose	Xylose	Galactose
Hemicellulose A	–	+	+	+
Hemicellulose B	–	+	–	–

It is probable therefore that hemicellulose A consists of about 11 % uronic acid anhydride, 79 % arabinose and xylose calculated as anhydroarabinose, and 10 % anhydrogalactose, while hemicellulose B yielding about the same percentage of furfuraldehyde contains 32 % uronic acid anhydride, and 68 % arabinose.

The nature and quantity of the furfuraldehyde-yielding substances in the Cross and Bevan cellulose.

It was next necessary to investigate the nature of the furfuraldehyde-yielding groups in the Cross and Bevan cellulose product. The cellulose fraction of straw isolated by this means is not unique in that it yields a considerable amount of furfuraldehyde, in this case over 11 %, since practically all Cross and Bevan celluloses appear to do this whatever their source. It has been customary arbitrarily to calculate the results as "pentosans" and to subtract this from the percentage of Cross and Bevan cellulose. This procedure has been criticised on the grounds that the furfuraldehyde may arise from sources other than pentosans, such as oxidation products formed from cellulose during the process of chlorination. This possibility was first examined.

The action of practically every oxidising agent on cellulose is the production of oxycelluloses, to a greater or less extent. This rather vague group has received the attention of many investigators, since the production of oxycellulose in the treatment of fibres and fabrics is accompanied by a loss of strength. Oxycelluloses contain an aldehydic group as evidenced by the reduction of Fehling's solution, and the formation of osazones. Further, they yield furfuraldehyde on distillation with 12 % HCl, the amount varying with the degree of oxidation employed in their preparation. More recently it has been shown by Heuser and Stöckigt [1922], and Hibbert and Parsons [1925] that carbon dioxide is evolved on suitable acid treatment, indicating the presence of glycuronic acid groups. This would explain the production of furfuraldehyde and led the workers named above to suggest that the process of production of oxycelluloses is accompanied by the progressive formation of uronic groups. It has not, however, been shown that the furfuraldehyde from oxycelluloses arises only from these uronic groups, and to throw some light on this point the yields of furfuraldehyde and CO_2 from the same preparations were determined.

Preparation	Source	Oxidising agent	Copper number	Furfuraldehyde yield %	CO_2 yield %	Uronic acid anhydride %	Furfuraldehyde due to uronic acid anhydride %
α-Oxycellulose	Cotton-wool	$KClO_3 + H_2SO_4$	4·8	0·80	0·62	2·48	0·41
β- ,,	,,	HNO_3	9·6	0·65	1·1	4·4	0·72
γ- ,,	,,	H_2SO_4 on β	9·4	0·40	0·66	2·63	0·42
δ- ,,	,,	NaOH to β filtrate	14·0	1·18	1·36	5·44	0·91

It will be seen that the furfuraldehyde due to the uronic acid is approximately the total furfuraldehyde given by the preparation within the limits of error of the latter estimation, so that it is clear that the sole result of oxidation is the production of uronic groups.

It was next important to determine the extent of the oxidation which takes place in the Cross and Bevan chlorination. To this end samples of cotton-wool and filter-paper were chlorinated for three periods of 12, 7 and 5 minutes, being treated with sulphite in the intervals as described previously. The yields of furfuraldehyde of the original sample and of the final chlorination product were determined. This process of chlorination results, therefore, in only a very small production of uronic groups, and the criticism that the furfuraldehyde-yielding substances found with the Cross and Bevan cellulose fractions are due to oxidation products is not justified.

Material	Recovery after chlorination, %	Furfurald. before chlorination, %	Furfurald. after chlorination, %
Cotton-wool	98·24	0·66	0·71
Filter-paper, Whatman No. 2	92·58	0·18	0·21
Filter-paper, Whatman No. 41	98·74	0·13	0·23

However, the precise nature of these substances in the cellulose is a difficult problem to settle. A determination of the yield of CO_2 from the chlorination product at once shows that it is not a hemicellulose similar to the free hemicelluloses isolated, for the content of uronic acid anhydride is extremely low.

	%
Furfuraldehyde yield of Cross and Bevan cellulose ...	11·32
CO_2 yield of Cross and Bevan cellulose	0·22
Uronic acid anhydride	0·88
Furfuraldehyde due to uronic acid anhydride	0·15

Two weighed portions of the chlorination product were extracted for 4 hours at 100° with 4 % NaOH. The alkali was partially neutralised and filtered. After washing, one portion was used for the determination of the yield of furfuraldehyde, and the other for that of CO_2.

	%
Furfuraldehyde yield of alkali-extracted cellulose	9·2
CO_2 yield of alkali-extracted cellulose	0·23

From the fact that the furfuraldehyde yield was lowered without affecting

that of CO_2, it is to be supposed that the pentose groups yielding furfuraldehyde are not combined with the uronic acid, though this supposition involves the doubtful assumption that this substance in the cellulose is homogeneous. It would seem not unlikely from the evidence that this small amount of CO_2 arises from a fraction of natural oxycellulose present, together with that formed by oxidation during the chlorination process. The alternative is that there is a small amount of hemicellulose similar in type to hemicellulose A and B.

It is not possible to prepare any large amount of the chlorinated product, so that any attempt at a preparation of the main furfuraldehyde-yielding substances in it is out of the question. Since the presence of hexosans in the Cross and Bevan cellulose fraction has been reported in the case of certain woods, a portion was boiled with 1 % sulphuric acid for several hours. After neutralisation the extract was tested for mannose and galactose without result. The pentose present appeared to be xylose. There is reason to suppose that very little is removed by this acid hydrolysis, and in consequence the result is not entirely conclusive. However, it seems not improbable that it is a xylan intimately associated with the cellulose which is responsible for this yield of furfuraldehyde. Irvine and Hirst [1924] have shown that esparto cellulose contains 81·5 % normal cellulose and 18·5 % xylan, while Heuser and Haug [1918] state that the cellulose from a cereal straw (type unspecified) contained as much as 22·34 % xylan. In the straw under investigation the pentose present calculated as anhydroxylose amounts to 17·35 % of the cellulose product.

The relationship of this xylan to the true cellulose has been a matter of considerable controversy, since the xylan appears to be so intimately associated that removal is a matter of considerable difficulty. Two extractions were carried out to investigate this point. In the first, 100 g. straw were treated in a boiling water-bath with 1 litre 0·5 % ammonium oxalate twice for a period of 1 hour each, to remove pectin. This was followed by four treatments of an hour with 4 % NaOH, and finally with water to ensure that all the alkali had been removed. The following analytical figures were obtained on this residue:

	C. and B. cellulose %	Total furfurald. %	Furfurald. on 100 g. C. and B. cellulose g.	Furfurald. on cellulose in 100 g. material g.	Carbon dioxide yield %	Uronic acid %
Oat straw	53·14	16·08	11·32	5·99	1·21	4·84
Residue	91·68	9·09	7·83	7·18	0·46	1·84
100 g. original straw	50·3	5·0	—	3·9	0·25	1·00

It was not possible to determine the actual loss during this treatment, since the alkaline extracts have to be filtered on cloths, during which some losses occur. It is, however, possible approximately to calculate this. The calculation depends on the assumption that there is no loss of true cellulose in this extraction. The total amount of true cellulose originally present was 53·14 %,

less the associated xylan which amounted to (5·99 × 1·55) %, 1·55 being the factor for obtaining anhydroxylose from the yield of furfuraldehyde. In the course of the extraction a certain portion of the xylan associated with the cellulose is removed. The amount remaining may be determined from the ratio of furfuraldehyde given by 100 g. Cross and Bevan cellulose after extraction to that given by 100 g. original product. This amounts to

$$\left[(5{\cdot}99 \times 1{\cdot}55) \times \frac{7{\cdot}83}{11{\cdot}32}\right].$$

The total residual straw may be designated by Q, 91·68 % of which is Cross and Bevan cellulose, composed of the true cellulose present originally in 100 g. straw, together with the residual associated xylan. Expressed in figures, this is

$$91{\cdot}68\,\frac{Q}{100} = \left[53{\cdot}14 - (5{\cdot}99 \times 1{\cdot}55) + \left\{\frac{(5{\cdot}99 \times 1{\cdot}55 \times 7{\cdot}83)}{11{\cdot}32}\right\}\right]$$

$$Q = 54{\cdot}8\ \%.$$

The loss during extraction is therefore 45·2 %. It is then possible from this to obtain the analytical figures of the residue calculated back on 100 g. of original straw. These are given in the third horizontal row of the table above. While the furfuraldehyde yield from groups unassociated with the cellulose has decreased from 10·08 % to 1·1 %, a loss of nearly 90 %, that from the xylan has fallen from 5·99 % to 3·9 %, a loss of only 35 %.

A second extraction which was carried out was very much more drastic. 100 g. of dried straw, finely ground, was treated on a boiling water-bath with, in turn, 1 litre 0·5 % ammonium oxalate for two periods of 4 hours each, 4 % NaOH for four periods of 4 hours each, and finally with water for two periods of 2 hours. The residue after drying gave the following analytical figures:

	C. and B. cellulose %	Total furfurald. %	Furfurald. on 100 g. C. and B. cellulose g.	Furfurald. on cellulose in 100 g. material g.
Oat straw	53·14	16·08	11·32	5·99
Residue	94·41	3·05	3·07	2·90
100 g. original straw	48·05	1·55	—	1·48

The loss during extraction was determined as described above from the expression

$$94{\cdot}41\,\frac{Q'}{100} = \left[53{\cdot}14 - (5{\cdot}99 \times 1{\cdot}55) + \left\{\frac{(5{\cdot}99 \times 1{\cdot}55 \times 3{\cdot}0)}{11{\cdot}32}\right\}\right]$$

$$Q' = 49{\cdot}1\ \%.$$

The loss was therefore 50·9 % indicating the removal of all but a trace of the furfuraldehyde groups unassociated with the cellulose, while the associated xylan sustained a loss of only 75 %.

In this property of great resistance to extraction the xylan associated with the cellulose of this straw is in no wise different from that of other straws and woods as noted by various investigators. Heuser and Haug [1918], when

employing reasonable methods of extraction, found it impossible completely to free from xylan the cellulose from a cereal straw. Six extractions with 6 % NaOH at 100° only resulted in a loss of 80 %, while the residue after treatment with NaOH under pressure at 140–150° retained about 30 % of the xylan. By very drastic treatment with 17 % NaOH in the cold it is possible to remove practically all of the furfuraldehyde-yielding substances, but at the same time the β- and γ-cellulose fractions are also removed.

The relationship of this associated xylan to the cellulose is a question upon which it is impossible to come to a decision. Cross and Bevan originally viewed as cellulose all the non-nitrogenous structural matter of plants, stating that there were many different forms of cellulose, both physical and chemical. It gradually became recognised that the cotton fibre was alone in consisting of cellulose almost solely, cellulose in this sense being a chemical entity built up of anhydroglucose groups. Other plant tissues were stated to be composed of a cellulosic group in attachment with lignin as "lignocelluloses," pectin as "pectocelluloses," hexosans as, for example, the mannocellulose of Schulze, with pentosans, and with other groups. Wislicenus and Kleinstück [1910] were the forerunners of the modern adsorption theory. Though applied by them to the process of lignification, it applies to all groups, since they viewed lignin as being the sum total of the hydrosols of the cell sap deposited on the cellulosic membranes by adsorption. Modern methods of attack have thrown more light on this problem. X-ray analysis employed by Hertzog and Jancke [1920] indicates that the cellulose from many plant resources is identical, while Irvine and Hirst [1924] by their methylation process have shown that the cellulose from esparto is chemically identical with cotton cellulose. Most investigators are now agreed in viewing cellulose as a large aggregate made up of many units each comparatively small in size, containing only a few anhydroglucose groups. These units are presumed to be held together by secondary valency, interference with which results in the alteration of the physical state and the degree of adsorption possible. It is also conceivable that other units, such as pentosans which have been formed during development in close association with the cellulose, may be present in the aggregate, being held by secondary valency. The type of these units found will depend on the conditions obtaining during growth, but the amount found may bear no relationship to the amount really present, since it depends entirely on the method of purification to which the cellulosic tissue has been subjected. Two methods of association are involved in this theory, adsorption and casual linkage by secondary or residual valency. Further, it is not impossible that the difficulty of extraction of these associated substances may also be in part a mechanical one. Furfuraldehyde-yielding substances appear very early in the development of a tissue and it is possible that in thickening cellulose may be deposited on the pentosan material. The middle lamella, originally pectic in nature, may be converted by decarboxylation into pentosan, which would be entirely "occluded" in the cellulose of the walls. Such "occluded" material,

even if in itself quite soluble, would be difficult of extraction until the cellulosic barrier had been ruptured or physically altered by drastic treatment such as gelatinisation by alkali. It is claimed that this may be in part a contributory factor in the apparent resistance of these substances associated with the cellulose aggregate.

Having thus considered the nature of the furfuraldehyde-yielding substances of this straw, it is possible to present a more rational balance sheet of the various constituents. The methods which must be followed to obtain these figures obviously prevent any claim of absolute accuracy, but it is claimed that they are a close approximation. It is certain that there is some overlapping, though it is not easy to see where this occurs unless it is in the lignin fraction. The method of Ost and Wilkening [1910] involving the use of 72 % H_2SO_4 gave a mean figure of 18·54 %, while the alkaline extraction method due to Mehta [1925] gave a figure for "lignic acid" of only 8·43 %. It is possible that an unduly high figure for lignin may be obtained for reasons connected with the structure of the tissue. It would not be unreasonable to picture a layer of lignin, either adsorbed, or attached by secondary valency and completely overlying a unit of cellulose. By the acid method the whole of this fragment might be returned as lignin even though the resistant lignin coating was but a fraction of the whole. The analyses are not complete, for no attempt has been made to determine accurately such groups as tannins, starch, and free sugars, since these were not of major importance in the fermentation studies to follow. Roughly, however, these last do not together account for more than 2 % of the whole. This method of evaluation of the furfuraldehyde-yielding constituents is applicable generally, and has been successfully applied in the case of certain woods.

It is now possible to summarise the various interpretations of the analyses of this straw originally quoted on p. 1356.

Composition of oat straw.

	%
Ash	7·07
Free hemicellulose	22·8
"Pure" cellulose	43·8
Xylan associated with cellulose	9·3
Pectin (calcium pectate yield)	1·1
Protein	1·8
Lignin (72 % H_2SO_4)	18·5

The same procedure was followed throughout in the case of rye straw.

Rye straw.

	%
Ash	3·46
Total furfuraldehyde yield	17·05
Carbon dioxide yield	1·15

Rye straw (continued).

	%
Calcium pectate yield	0·34
Cellulose (Cross and Bevan)	55·27
Furfuraldehyde from cellulose	5·64
Lignin	19·5
Protein	1·9

Furfuraldehyde arising from the pentose groups of the hemicelluloses present in 100 g. straw is given by

$$[A - \{B + D + (C - E)\}],$$

whence

$$[17{\cdot}05 - \{5{\cdot}64 + 0{\cdot}77 + (0{\cdot}07 - 0{\cdot}04)\}] = [17{\cdot}05 - 6{\cdot}44] = 10{\cdot}61\ \%.$$

Mixed hemicellulose preparation.

	%
Furfuraldehyde yield on ash-free basis ...	34·0
CO_2 yield on ash-free basis	3·3
Uronic acid anhydride	13·2
Furfuraldehyde due to uronic groups ...	2·2

By difference, furfuraldehyde due to pentose units of hemicellulose 31·8 %. The free hemicellulose content of this straw is therefore approximately 33·4 %.

Purification of the mixed hemicellulose product was carried out.

Rye straw.

	Hemicellulose A %	Hemicellulose B %
Furfuraldehyde yield on ash-free basis...	32·7	37·1
CO_2 yield on ash-free basis	1·29	7·1
Uronic acid anhydride	5·16	28·4
Furfuraldehyde due to uronic groups ...	0·86	4·7
Furfuraldehyde due to pentose groups...	31·8	32·4

The nature of the sugars present in these products was not determined. Hemicellulose A contains however approximately 5 % uronic acid anhydride, 60 % anhydropentose (calculated as arabinose) and 35 % anhydrohexose. Hemicellulose B is composed of approximately 28·5 % uronic acid anhydride, 60·5 % anhydropentose (calculated as arabinose) and 11 % anhydrohexose.

The furfuraldehyde-yielding substance associated with the cellulose appeared in this case also to be xylan. In amount it is present to the extent of 15·82 % of the Cross and Bevan cellulose fraction, or 8·74 % of the straw. As it will be seen from the analytical figures quoted below, the xylan was even more difficult of extraction than that of oat straw. Two series of extractions were carried out, the first with 4 % NaOH for four periods of 4 hours each, and the second similarly but preceded by two treatments of 2 hours each with 1 % NaOH in 55 % alcohol.

	C. and B. cellulose %	Total furfurald. yield %	Furfurald. on 100 g. C. and B. cellulose g.	Furfurald. on cellulose in 100 g. material g.
Rye straw	55·27	17·05	10·21	5·64
Extraction I	90·01	5·73	5·57	5·01
Extraction II	91·56	5·50	5·57	5·10

By calculation the loss in the case of extraction I was approximately 43 %, and in the case of extraction II, 44 %. It was possible therefore to determine the true loss of each group as determined on 100 g. of the original material.

	C. and B. cellulose %	Total furfurald. yield %	Furfurald. on C. and B. cellulose in 100 g. material g.
Preparation I	51·30	3·27	2·86
Preparation II	51·24	3·07	2·85

In the case of the more drastic extraction, the furfuraldehyde yield from groups unassociated with cellulose has fallen from 11·41 to 0·22, a loss of 98 %; while that of the xylan has fallen from 5·64 to 2·85, a loss of only 50 %. The xylan associated with the cellulose of oat straw sustained a loss of 75 % under the same treatment. There is obviously therefore a considerable difference either in the physical condition or method of linkage of this substance in these two straws, which must be considered later when the availability to micro-organisms is being discussed.

It will be convenient now to re-state the results obtained for the composition of this straw. Overlapping was again evident, though not possible to place, unless again it occurred in the lignin fraction.

Composition of rye straw.

	%
Ash	3·46
Free hemicellulose	33·40
"Pure" cellulose	46·53
Xylan associated with cellulose	8·34
Pectin (calcium pectate yield)	0·34
Protein	1·9
Lignin (72 % H_2SO_4)	19·50

SUMMARY.

1. Figures quoted for "pentosan" obtained by use of the Kröber factor on total furfuraldehyde yield are unreliable because of the uronic and hexose groups in the hemicelluloses, and the furfuraldehyde-yielding groups intimately associated with the Cross and Bevan cellulose fraction.

2. If an adjustment is made for these and for the pectin present, it is possible to obtain a satisfactory figure for furfuraldehyde due only to the pentose units of the hemicelluloses.

3. An approximation for the actual content of hemicelluloses in the

material may then be obtained by determining the furfuraldehyde yield from the pentose groups in a mixed hemicellulose product obtained by alkaline extraction.

4. Oat straw is shown to contain approximately 22·8 % free hemicelluloses, being a hemicellulose A consisting of about 11 % uronic acid anhydride, 79 % arabinose and xylose (expressed as anhydro-arabinose) and 10 % anhydro-galactose, and a hemicellulose B yielding about the same percentage of furfuraldehyde but containing 32 % uronic acid anhydride and 68 % arabinose.

5. The furfuraldehyde-yielding substance associated with the Cross and Bevan cellulose fraction in both oats and rye is shown not to consist of oxidation products arising from the chlorination process, but to be due to xylan free from uronic groups. This xylan is extremely resistant to extraction and is presumably held by the cellulose aggregate by secondary valency. It is suggested that the difficulty of extraction may be, in part at least, a mechanical phenomenon, a portion of the pentose being "occluded" during development.

6. Rye straw is shown to contain about 33 % of free hemicelluloses, the hemicellulose A fraction being built up of approximately 5 % uronic acid anhydride, 60 % anhydropentose, and 35 % anhydrohexose, while the hemicellulose B fraction contains roughly 29 % uronic acid anhydride, 60 % anhydropentose, and 11 % anhydrohexose.

The writer wishes to express his indebtedness to Sir John Russell, Director of the Rothamsted Experimental Station, for placing at his disposal the facilities of the Station; and to the Department of Scientific and Industrial Research for a Senior Research Award, during the tenure of which this work was done. His thanks are especially due to Mr E. H. Richards, Head of the Fermentation Department whose ready advice and assistance throughout have been invaluable.

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CXLVIII. THE BIOLOGICAL DECOMPOSITION OF PLANT MATERIALS.

II. THE RÔLE OF THE FURFURALDEHYDE-YIELDING SUBSTANCES IN THE DECOMPOSITION OF STRAWS.

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(*Received November 1st, 1929.*)

Introductory.

That the decomposition of cellulosic materials, whether in the soil, the manure heap, or in composts, is a microbiological process has long been recognised, but it sometimes appears to be overlooked that this process is not a single action, but the resultant of the metabolic activities of many organisms, the microflora varying according to the conditions obtaining. Although the morphological characters of many organisms participating in these processes have been described, in but few cases have their particular biochemical actions on the plant constituents been determined. In this work it is proposed to study first the general process of decomposition, and then the biochemical activities of some of the more important organisms which are found.

It is obvious that the microbiological decomposition of a particular plant tissue depends on the availability of its chemical components to the organisms present. If it is to be decomposed rapidly there must be present available carbonaceous material both to meet the energy requirements, and to build up the necessary protoplasm. At the same time there must be a sufficiency of nitrogen to meet the structural demands of the micro-organisms in building up biological protein. Mature plant materials tend usually to contain rather a low percentage of nitrogenous substances, and many investigators have shown that the amount of nitrogen available controls the rate and degree of decomposition by micro-organisms, provided that the carbonaceous constituents of the material are at all available to the organisms concerned. The nitrogen is immobilised temporarily by conversion into protein and allied nitrogenous compounds, in the protoplasm of the organisms. In this immobilisation of nitrogen, and, to a lesser extent, the immobilisation of other necessary inorganic ions, lies the explanation of the injurious effect of the addition of straw to the soil on the growth of the crop following. Hutchinson and Richards [1921], in their work on the production of artificial manure from vegetable materials, recognised that simple nitrogenous substances were essential for this decomposition, and determined the quantity necessary for a satisfactory

rot under normal aerobic conditions. They found that for 100 g. of straw about 0·7 g. of nitrogen in a simple form was necessary for rapid decomposition. Moreover they found that the addition of an excess of nitrogen retarded very considerably the rate and extent of this process, 2 % being apparently the upper limit. In their patent for the making of artificial manure (Adco) Richards and Hutchinson [1924] claimed that materials "which contain an adequate total quantity of carbohydrate (for example, 30 % upwards) such as starch or pentosans, and preferably not too high a proportion of lignocellulose" were capable of undergoing decomposition in this way. This patent appears to contain the first record of an attempt to account for the decomposability of any tissue in terms of the substances composing it.

Hebert [1892] had examined the order of decomposition of the constituents of straw and stated that vasculose (lignin) was the most resistant. This was borne out by Dvörak [1912], who found that mature and old plants decomposed less readily than young plants owing to the resistant lignocellulose in the former. In connection with his work it must be remembered that the nitrogen content of the young plants is as a rule much higher than in the same plant at maturity.

Accepting therefore the importance of nitrogen in this process, the problem of decomposition of vegetable materials appears to be bound up with the chemical composition of the plant, and the availability of its constituents to the microflora present. This aspect of the question was investigated by Rege [1927] (see p. 1378).

Waksman and Tenney [1926, 1927, 1, 2, 1928], in investigating the formation of humus, come to the conclusion that it is the lignin fraction which is the most resistant constituent of plant tissues, and the one which accumulates during the process of rotting. The soluble materials, sugars, starch, etc., the cellulose, and pentosans are progressively removed.

Scheme of work.

The investigations to be described are centred round the furfuraldehyde-yielding constituents, and the relative importance of these substances in the rotting of plant materials in the presence of sufficient available nitrogen. Since it is possible to differentiate between the different furfuraldehyde-yielding substances as described in the preceding paper, an examination is made of the theory put forward by Rege [1927] of the rôle of these substances, grouped together by him as "pentosans." In all the experiments described herein the rotting was effected by a mixed natural flora. More critical studies are in progress in which the biochemical effects of single organisms isolated from the natural flora are being determined.

Experimental.

The materials used in these preliminary investigations were straws, since these are known to rot comparatively easily under normal conditions. Two

were selected, oat straw, which decomposes readily, and rye straw which appears in practice to be somewhat more resistant. Detailed analyses of each of these are given in the preceding communication [Norman, 1929]. So that the rotting might be as complete as possible the optimum conditions were studied and adopted. The straws were chaffed for convenience in handling and to ensure proper wetting. Equal amounts of straw of known moisture content were weighed out and moistened thoroughly by spraying, being then bottled. Available nitrogen was added in the form of ammonium carbonate solution, to the extent of 1 g. nitrogen to 100 g. straw. The bottles were then laid down in an incubator at 35°, and frequently turned, the contents being stirred up periodically for the first few days to ensure even distribution of the nitrogen and uniform wetting. It is important that the straw be kept thoroughly wet throughout the process, additional water being supplied by a spray when necessary. Water-logging, however, must at all costs be avoided, since anaerobic conditions then set in, and the whole course of the process is altered.

In the case of the oat straw fermentations, an inoculum from old well-rotted manure was added to each, but later it was found that this is quite unnecessary since chaffed straw itself commonly carries an extensive and efficient microflora. The rye straw fermentations therefore did not receive any inoculum.

Every four days a bottle was taken for the dry weight determination. The following analyses were carried out on the dried residue in each case, the methods employed being precisely as described in the preceding paper [Norman, 1929].

(i) Ash.
(ii) Total furfuraldehyde yield.
(iii) Cellulose (Cross and Bevan).
(iv) Furfuraldehyde from Cross and Bevan cellulose.
(v) Calcium pectate yield.
(vi) Carbon dioxide yield (uronic acids).

Mycelial hyphae were observed by the sixth day, remaining visible till about the twentieth day. In the case of large bottles containing a considerable quantity of straw larger forms, such as *Coprinus*, sometimes appeared at the end of five or six weeks. In no case, however, did this occur in the smaller bottles as used for analysis.

The analytical figures obtained for Series I (oat straw) and Series II (rye straw) are given in Tables I and II; they are all calculated on the basis of 100 g. of the original straw employed.

It was shown in the preceding paper that it is possible to obtain from these figures considerable information as to the quantity of the various furfuraldehyde-yielding substances present. The groups upon which light is thrown and the method by which they are arrived at are as follows.

(i) *Total uronic acid anhydride.*

CO_2 yield $\times$ 4.

(ii) *Uronic acid anhydride in pectin.*

Calcium pectate yield $\times \frac{70}{100}$.

(iii) *Non-pectin uronic acid, i.e. in hemicelluloses.*

Difference of above.

(iv) *Furfuraldehyde from total uronic acid (D).*

Total uronic acid $\times \frac{16{\cdot}66}{100}$.

(v) *Furfuraldehyde from pectin (C).*

Calcium pectate yield $\times \frac{19{\cdot}8}{100}$.

(vi) *Furfuraldehyde from uronic acid in pectin (E).*

Uronic acid in pectin $\times \frac{16{\cdot}66}{100}$.

(vii) *Furfuraldehyde from pentose in pectin.*

$(C - E)$.

(viii) *Furfuraldehyde from pentose groups in hemicelluloses.*

$[A - \{B + D + (C - E)\}]$

where A = total furfuraldehyde yield,
B = furfuraldehyde from C. and B. cellulose.

(ix) *Xylan associated with C. and B. cellulose.*

Furfuraldehyde in C. and B. cellulose $\times$ 1·55.

N.B. This factor 1·55 converts furfuraldehyde to anhydroxylose, and is applied in the case of the two straws under investigation since it has been shown that it is xylan that is present. In other materials it may not be xylan, and in consequence another factor will have to be found.

(x) "*Pure*" *cellulose.*

(C. and B. cellulose – associated xylan.)

Table I. *Decomposition of oat straw at* 35°.

(Expressed on 100 g. original dry straw.)

Day	Dry matter	Organic matter	Total furfurald. yield (A)	C. and B. cellulose	Furfurald. yield from C. and B. cellulose (B)	Calcium pectate yield	CO_2 yield	Lignin by 72 % H_2SO_4
—	100·0	92·93	16·08	53·14	5·99	1·10	1·21	18·5
4	92·3	85·11	12·29	52·13	6·00	1·16	1·20	—
8	85·5	78·41	9·96	39·63	4·51	1·43	1·14	—
12	76·5	69·13	9·16	34·37	4·06	1·52	1·03	—
16	72·5	65·43	8·86	31·09	3·90	1·53	1·06	—
20	69·2	62·16	9·37	28·54	3·83	1·56	0·97	—
24	66·3	59·19	9·24	27·04	3·77	1·66	0·89	—
28	65·4	58·28	8·92	25·21	3·57	1·80	0·89	—
32	64·7	57·66	7·67	25·02	3·47	1·70	0·92	—
36	64·0	56·98	6·77	23·70	3·29	1·77	0·92	—
40	63·5	56·45	6·51	22·58	2·95	1·76	0·87	—
56	61·0	53·92	6·12	21·67	2·71	1·76	0·65	—
85	55·9	48·85	5·59	19·91	2·29	1·73	0·65	—

Table II. *Decomposition of rye straw at* 35°.

(Expressed on 100 g. original dry straw.)

Day	Dry matter	Organic matter	Total furfurald. yield (A)	C. and B. cellulose	Furfurald. yield from C. and B. cellulose (B)	Calcium pectate yield	CO_2 yield	Lignin by 72 % H_2SO_4
—	100·0	96·54	17·05	55·27	5·64	0·34	1·15	19·5
4	96·9	93·46	15·07	54·09	5·68	0·34	1·08	—
8	79·1	75·61	10·73	40·86	5·56	0·40	0·79	—
12	72·4	68·92	10·02	36·89	5·02	0·43	0·81	—
16	68·5	65·00	8·38	32·56	3·89	0·50	0·76	—
20	66·2	62·73	7·69	31·41	3·72	0·54	0·72	—
24	63·1	59·67	7·21	29·48	3·35	0·60	0·66	—
28	60·4	56·94	7·63	28·77	3·35	0·70	0·71	—
33	59·7	56·20	7·36	27·58	3·20	0·73	0·69	—
36	59·3	55·84	7·26	27·32	3·21	0·77	0·70	—
40	58·4	54·96	7·26	26·77	3·18	0·80	0·70	—
48	56·9	53·35	7·22	26·41	3·22	0·80	0·71	—
84	50·5	47·01	5·75	23·17	2·67	0·82	0·51	—

Table III. *Decomposition of oat straw at* 35°.

Furfuraldehyde-yielding constituents and cellulose.

Day	Total uronic acid anhydride	Uronic acid in pectin	Non-pectin uronic acid	Furfurald. from total uronic acid (*D*)	Furfurald. from pectin (*C*)	Furfurald. from uronic acid in pectin (*E*)	Furfurald. from pentose in pectin (*C*−*E*)	Furfurald. from pentose groups in hemi-celluloses [*A* −{*B* + *D* + (*C* − *E*)}]	Xylan associated with C. and B. cellulose	"Pure" cellulose by difference
—	4·84	0·77	4·07	0·81	0·21	0·13	0·08	9·2	9·28	43·86
4	4·80	0·81	3·99	0·80	0·23	0·14	0·09	5·4	9·3	42·83
8	4·56	1·00	3·56	0·76	0·28	0·17	0·11	4·6	7·0	38·63
12	4·12	1·06	3·00	0·68	0·29	0·18	0·11	4·4	6·3	31·07
16	4·24	1·07	3·17	0·71	0·30	0·18	0·12	4·1	6·1	27·59
20	3·88	1·09	2·79	0·65	0·31	0·18	0·13	4·8	6·0	23·54
24	3·56	1·16	2·40	0·59	0·32	0·19	0·13	4·7	5·9	21·14
28	3·56	1·26	2·30	0·59	0·35	0·21	0·14	4·6	5·6	19·61
32	3·68	1·19	2·50	0·61	0·33	0·20	0·13	3·5	5·4	19·62
36	3·68	1·23	2·45	0·61	0·34	0·21	0·13	2·8	5·1	18·60
40	3·48	1·23	2·25	0·58	0·34	0·20	0·14	2·8	4·6	17·98
56	2·60	1·23	1·37	0·43	0·34	0·20	0·14	2·8	4·2	17·47
85	2·60	1·21	1·39	0·43	0·33	0·20	0·13	2·7	3·6	16·31

Table IV. *Decomposition of rye straw at* 35°.

Furfuraldehyde-yielding constituents and cellulose.

Day	Total uronic acid anhydride	Uronic acid in pectin	Non-pectin uronic acid	Furfurald. from total uronic acid (*D*)	Furfurald. from pectin (*C*)	Furfurald. from uronic acid in pectin (*E*)	Furfurald. from pentose in pectin (*C*−*E*)	Furfurald. from pentose groups in hemi-celluloses [*A* −{*B* + *D* + (*C* − *E*)}]	Xylan associated with C. and B. cellulose	"Pure" cellulose by difference
—	4·60	0·24	4·36	0·77	0·07	0·04	0·03	10·6	8·7	46·54
4	4·32	0·24	4·08	0·72	0·07	0·04	0·03	8·6	8·8	45·30
8	3·16	0·28	2·88	0·53	0·08	0·05	0·03	4·6	8·6	32·24
12	3·24	0·30	2·94	0·53	0·09	0·05	0·04	4·4	7·8	29·11
16	3·04	0·35	2·69	0·51	0·10	0·06	0·04	3·9	6·0	26·60
20	2·88	0·38	2·50	0·48	0·11	0·06	0·05	3·4	5·7	25·66
24	2·64	0·42	2·22	0·44	0·12	0·07	0·05	3·4	5·2	24·29
28	2·84	0·49	2·35	0·47	0·14	0·08	0·06	3·7	5·2	23·58
33	2·76	0·51	2·25	0·46	0·14	0·08	0·06	3·6	5·0	22·62
36	2·80	0·54	2·26	0·47	0·15	0·09	0·06	3·5	5·0	22·35
40	2·80	0·56	2·24	0·47	0·16	0·09	0·07	3·6	4·9	21·84
48	2·84	0·56	2·28	0·47	0·16	0·09	0·07	3·5	5·0	21·42
84	2·28	0·57	1·71	0·38	0·16	0·10	0·06	2·6	4·1	19·03

These various calculations were made on the results of each series, and are quoted in Tables III and IV.

All figures of special significance are graphed below. In each case time is plotted against absolute loss, not percentage loss, on the amount of the constituent originally present in 100 g. dry straw. Figs. 1 and 4 deal with the loss of major constituents in oats and rye respectively, 2 and 5 the fall in furfuraldehyde yields, and 3 and 6 the loss in uronic contents.

Discussion of results.

For the sake of clarity it will be advisable to confine this discussion first to the figures obtained for the decomposition of oat straw, as given in Tables I and III and Fig. 1.

The most prominent feature of the decomposition of this straw is not so much the very rapid loss of organic matter, amounting to one-third of the whole in 24 days, but the loss in this time of 50 % of the cellulose of the straw, equivalent in quantity to two-thirds of the lost organic matter. At the close of the period of 2 months there is a loss of 42 % of the organic matter, the loss of cellulose over the same period being 60 % of that originally present. The cellulose lost accounts for nearly 70 % of the lost organic matter. It is perfectly clear from this, and from other observations recorded that the most important feature of this type of decomposition is the loss of cellulose. There may be other constituents which sustain a greater percentage loss, but nevertheless, the actual bulk loss of cellulose is by far the greatest.

On Fig. 1 there is plotted in dotted line the values for fall in furfuraldehyde yield due to pentose groups in the hemicelluloses. In the previous paper [Norman, 1929] it was shown that the hemicelluloses originally present in oats yielded from their pentose groups 40·4 % of furfuraldehyde so that the 9·2 % of furfuraldehyde from this source in 100 g. of straw represents a hemicellulose content of 22·8 %. It is not, however, justifiable to apply this factor for obtaining hemicellulose content to the analyses throughout the decomposition, since it is certain that it is only a mean figure from a mixture of various individual hemicelluloses, some of which may be more readily available than others, and consequently more quickly removed, thereby causing that factor to vary somewhat. However, considering for this purpose the furfuraldehyde yield to be an approximate measure of hemicellulose content, it is observed that there is over the whole period of 2 months a loss of nearly 70 % of these substances. The significant point, however, is that more than 40 % is removed in the first 4 days and a further 10 % in the next period of 4 days.

The removal of the xylan associated with cellulose appears to be slow and fairly constant in rate. In 2 months just over 70 % of the total amount present is lost. The rate is presumably controlled by the rate of removal of the cellulose.

Similar features are observed in the decomposition of rye straw as given

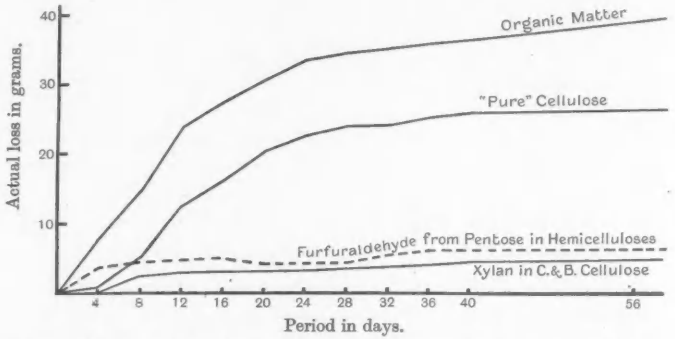

Fig. 1. Decomposition of oat straw at 35°. (Expressed on 100 g. original straw.)

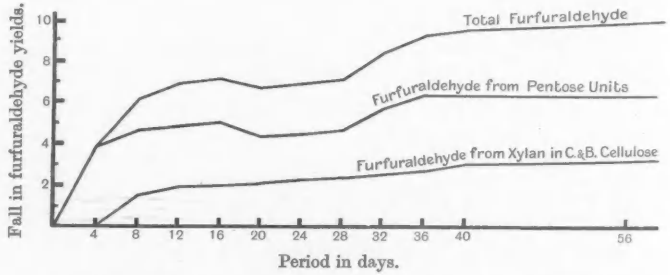

Fig. 2. Decomposition of oat straw at 35°. Fall in furfuraldehyde yields. (Expressed on 100 g. original straw.)

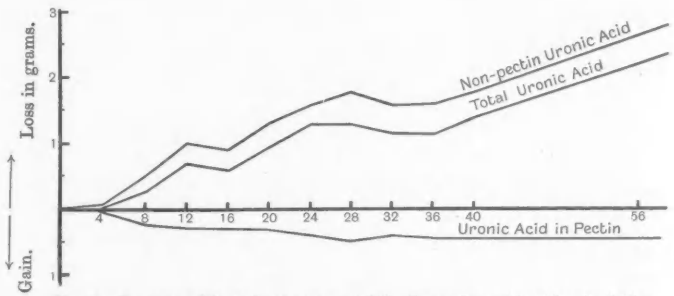

Fig. 3. Decomposition of oat straw at 35°. Loss of uronic acid anhydride. (Expressed on 100 g. original straw.)

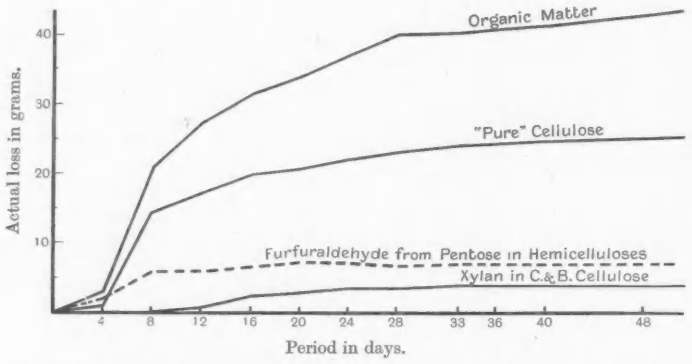

Fig. 4. Decomposition of rye straw at 35°. (Expressed on 100 g. original straw.)

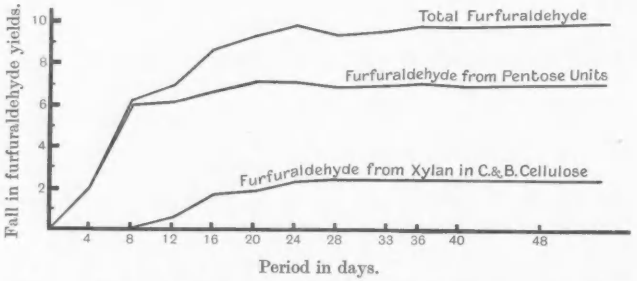

Fig. 5. Decomposition of rye straw at 35°. Fall in furfuraldehyde yields. (Expressed on 100 g. original straw.)

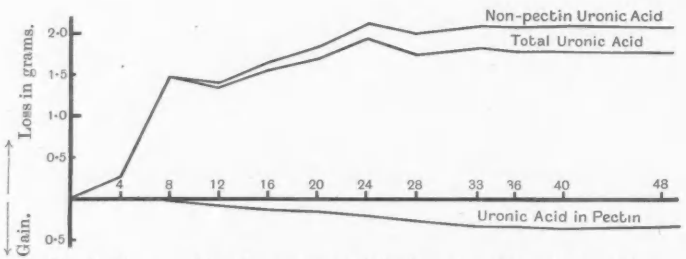

Fig. 6. Decomposition of rye straw at 35°. Loss of uronic acid anhydride. (Expressed on 100 g. original straw.)

in Tables II and IV and Fig. 4. The early stages in this case are not so rapid as in oat straw since it is a much more difficult matter to get proper wetting of rye straw. The loss over a period of 2 months is slightly greater than the loss of oat straw in the same period, being about 43 %. This is somewhat surprising in view of the fact that rye straw is always considered to be more resistant than oat straw, which is regarded as the cereal straw that is most easily decomposed. Again the rapid loss of cellulose is noteworthy. In 2 months 54 % of that originally present is lost, and of this 70 % is removed during the first 12 days.

The loss of hemicelluloses as measured by the furfuraldehyde due to their pentose units is also extremely rapid in the early stages, just as occurred in the case of oats. A loss of 57 % is observed in the first 8 days, the total over 2 months being only 69 %.

The xylan associated with cellulose appears to be removed at a fairly constant rate. Over the period of 2 months a loss of about 45 % of the total amount is sustained. This is considerably less than is observed in the case of oat straw.

To sum up then, the most obvious feature in each case is the rapid loss of the major portion of the hemicelluloses, followed by an equally rapid loss of cellulose. The hemicelluloses, however, appear to remain at much the same level after the first 12 or 14 days, while the cellulose continues progressively to decrease. There is some evidence that this former observation may possibly be more apparent than real.

To regard the hemicellulose present in any plant material as a homogeneous substance is to be in error. As has been shown in the case of these straws, there are two or more types present, separable by differences in physical behaviour. These two types, called A and B, are probably themselves mixtures also. All that it is possible to say is that they are built up on the same general plan, from pentoses, hexoses, and uronic acids, and possess some physical properties in common. Of the method of linkage nothing is known. Since the hemicelluloses of a plant tissue are non-homogeneous chemically it is unjustifiable to regard them as of equal biological value. Not only must the availability vary considerably, but some organisms seem incapable of breaking down certain pentose sugars and pentose groupings. Further there is more than a possibility that certain groups only of the hemicelluloses might be removed leaving new intermediate residues. The microbiological decomposition of this type of substance is an unexplored field.

In Fig. 2 various furfuraldehyde yields are plotted for the decomposition of oat straw. The fall of total furfuraldehyde yield is extremely rapid during the first 8 or 12 days as has been seen. After that period there is, however, a small but none the less definite increase, indicated by a depression in the curve for fall of furfuraldehyde yields. The whole of the furfuraldehyde lost during the first 4 days is seen to be due to the loss of pentose groups in the hemicelluloses. The increase of furfuraldehyde-yielding substance which occurs

later is shown also to be due to pentose groups apparently formed by the organisms from some other substance more rapidly than they are removed. The same features are visible in Fig. 5 for rye, though they are there not quite so well marked. These observations make it clear that the curve representing furfuraldehyde loss from pentose units applies to loss of original hemicelluloses only in the opening stages. The pentose units present after that period must consist of those of the original hemicelluloses as yet unattacked together with pentose units produced by biological action on some other grouping. In the later stages it is not improbable that the furfuraldehyde due to pentose units is almost entirely due to hemicelluloses or hemicellulose residues which are biologically not readily available, the accumulation of transitionally formed pentose units being removed as the rate of decomposition slackens.

The source of these transitory substances cannot be definitely stated, though in all probability they arise from the cellulose, of the route of the biological decomposition of which little is known. It is unreasonable to suppose that such a complex substance as cellulose must be broken down swiftly and completely to carbon dioxide and water without the appearance of any intermediate product. Most theories of cellulose fermentation [see Buchner and Meisenheimer, 1908] postulate a breakdown to cellobiose, glucose and thence to lactic acid from which various acids and aldehydes are formed. There is, however, an absence of evidence for such a route. Cellulase must be regarded not as a single enzyme but as a team of enzymes. Possibly in a rapid decomposition such as this, the cellulose may be destroyed in part symbiotically. There is some evidence supporting the production of some intermediate substance in cellulosic decomposition. The aerobic organism *Spirochaeta cytophaga* described by Hutchinson and Clayton [1919] decomposed cellulose readily with the formation of fatty acids, a yellow pigment, and a mucilaginous substance, non-reducing in character. The physical behaviour of this substance, together with the fact that it was definitely not of a dextrin nature, caused them to suggest that it resembled the pectic substances. In point of fact their evidence fits more closely the hemicellulose group, but it is possible that there is present a mixture of several degradation products. This paper contains the first clear reference to the production of intermediary substances in the decomposition of cellulose. Indirect evidence exists pointing to the same conclusion, but throwing no light on the nature of the substances concerned. In the decomposition of woods, as the cellulose is removed, there is a marked increase in alkali-soluble constituents. Although alkali-solubility seems always to be determined in studies on wood, no record exists of an attempt to determine the nature of the portion removed.

It may be, to take an extreme view, that all the hemicelluloses found at the close of active rotting are not constituents of the original material, but that they have been formed as intermediary compounds in the breakdown. Possibly even the furfuraldehyde does not arise from transitional pentoses at all but from some other grouping yielding furfuraldehyde under the conditions

of that estimation. If this is the case it would be expected that these transitional substances would disappear if the decomposition is greatly prolonged. However the losses of furfuraldehyde-yielding constituents under such conditions are only slow, and comparable with the loss of cellulose. It seems more satisfactory to view this residue of furfuraldehyde-yielding constituents as biologically less available either for constitutional or physical reasons. An investigation of the nature of these substances remaining at the end of several months is being undertaken with a view to throwing light on this point. Attempts will also be made to isolate any intermediary compounds which may be present earlier in the decomposition.

Other features of interest in the decomposition are shown in Figs. 3 and 6 in which the losses of uronic acid anhydride contents are plotted. In each case the total loss of uronic acid is steady and slow. The variations which are observed are within the limits of error of this determination. The most interesting point, perhaps, is that the pectin of the straw does not appear to be removed during the decomposition. In fact the figures would seem to indicate that there is a definite synthesis. Whether pectin itself is produced, or some other substance which is estimated as pectin under the conditions of that determination, it is not possible to say. The percentage content in oat straw rose from 1·1 to 1·7 in just a month, and seemed to remain constant at that. In rye which contains considerably less, the final figure is 0·8. Corrections in furfuraldehyde yield have been made on the assumption that it is pectin that is formed, but whether this is justifiable or not it is impossible to say. The corrections applied for this in the case of rye straw are hardly significant, but are applied to demonstrate the method. There is some evidence that the pectin is destroyed if the reaction of the rot becomes acid—an abnormal condition. Certainly the organisms present appear to decompose pure pectin actively, but not if the medium is kept neutral by the addition of chalk.

The loss of non-pectin uronic acid is presumably due to the degradation of the uronic groups in the hemicelluloses. That it is as small as it is would seem to support the theory mentioned above that there is a considerable residue of unavailable hemicellulose. If that is not so, then the uronic acid portion of the molecule must itself be less readily decomposed than the remainder, and intermediate compounds of high uronic acid content must be left.

General discussion.

From the results cited here, and from an analysis of those given by Rege [1927] and Waksman [1926, 1, 2; Waksman and Tenney, 1926; 1927, 2; 1928] it becomes clear that the theory of decomposition of cellulosic material as put forward by Rege is no longer tenable. Since the removal of cellulose is the most important feature of the process, and since the major loss of carbonaceous material is loss of cellulose, the cellulose must obviously be regarded as the most important food material, playing the chief part in the conversion of inorganic nitrogen to microbial protein. Rege's contention

that the large loss of cellulose is apparent, not real, cannot be justified by his own figures. It is true that the chlorination method of estimation of cellulose is not entirely satisfactory, but the error can only be small. Waksman and Tenney [1927, 1] who used a cuprammonium method for this estimation give figures in every way paralleled by those from the chlorination method. Observing the rapid decomposition of hemicelluloses which takes place in the first few days, and having proved that these substances are, unlike cellulose, readily available to the organisms in pure culture, Rege assumes that their presence is necessary as food, and that they therefore control the decomposability of the material. He is undeniably correct in regarding lignin as most resistant to biological attack and therefore the lignin content as a factor militating against decomposition. Rege connects the two factors, hemicellulose and lignin, and states that it is possible to predict the decomposability of any material by determining these two substances. If the ratio of food or energy factor, as he termed the hemicelluloses, to the inhibitory or lignin factor is greater than unity the material is easily decomposed, or if less than 0·5 it is very resistant. Such a theory ignores entirely the cellulose which forms a large part of plant tissues, and, as has been shown, forms the chief source of carbonaceous material for the organisms decomposing it. Moreover it fails to take into account mechanical and physical differences in structure of the tissues concerned. It is obvious that it is not possible to insert a factor into any formula to cover this variable quantity, but nevertheless it must be recognised as existing. Lignin being of a resistant nature acts as a physical barrier to the organism in search of food, and therefore the quantity present is an important factor, but no less important is its distribution, which cannot obviously be ascertained by any chemical means. The nearest one can get to a general statement is to say that the presence of a considerable quantity of cellulose and available hemicelluloses favours the decomposition of a tissue, while the presence of increasing quantities of lignin retards it.

The advisability of fixing a numerical relationship between these two is a matter upon which it is difficult to come to a decision. If it is recognised that structural and physical conditions are variables which it is not possible to assess, and that any decision on the basis of chemical determinations is subject to this other factor, then there can be no serious objection to its employment. With the above reservation it may be stated that if the ratio of cellulose and hemicelluloses to lignin is well over 3 the material will decompose fairly readily. If, however, it is in the neighbourhood of 2 decomposition will be very slight or non-existent. The following figures may be quoted.

Material	C. and B. cellulose + hemicelluloses	Lignin	Ratio
Oat straw	53·14 + 22·8 = 75·94	18·5	4·1
Rye straw	55·27 + 33·4 = 88·67	19·5	4·5
Deal	66·24 + 4·3 = 70·54	27·6	2·5

The figures for the two straws indicate clearly the importance of structural and physical considerations as stressed above. It might be supposed that rye straw decomposed more easily than oat straw, which in point of fact is not

the case. Oat straw decomposes with great ease, while rye straw in practice presents considerable difficulty, owing to its stiff waterproof nature.

Attempts have been made to provide further support for this theory by varying artificially the proportions of these groups in a natural material. That the hemicelluloses are not of pre-eminent importance is stressed by the work just to be described. Rye straw, which had been extracted to remove the hemicelluloses as completely as possible, was rotted down rapidly, though cellulose and lignin only were present. It is true that the lignin content was also lowered by the alkali treatment, but nevertheless, in accordance with the theory put forward by Rege [1927], the material should have been undecomposed.

Experimental.

Chaffed rye straw, as used for the above investigations, was extracted with hot water, and then four times for 4 hours each time with 4 % sodium hydroxide. It was very thoroughly washed and allowed nearly to dry, being broken up thoroughly. Equal portions were weighed out into bottles and moistened with a mixed mineral salt solution, since it was supposed that the alkali treatment would have removed much of this. Inorganic nitrogen was added as before, as ammonium carbonate, and an inoculum from rotting rye straw. The bottles were incubated at 35° and one taken for analysis every 8 days. The analytical figures obtained are given in Tables V and VI and illustrated in Figs. 7 and 8.

Table V. *Decomposition at 35° of rye straw extracted to remove hemicellulose and pectin.*

(Expressed on 100 g. rotted material.)

Day	Dry matter	Ash	Total furfurald. yield	C. and B. cellulose	Furfurald. yield on 100 g. C. and B. cellulose	Furfurald. yield on cellulose present	Lignin by 72 % H_2SO_4
—	100·00	1·48	5·73	90·01	5·57	5·01	9·3
8	89·02	1·66	4·05	87·44	3·67	3·21	—
16	82·71	1·79	3·90	83·90	3·75	3·15	—
24	76·49	1·92	3·50	82·68	3·25	2·79	—
32	73·99	2·00	3·30	80·04	3·48	2·79	—
48	68·85	2·15	3·20	75·97	3·10	2·36	—

Table VI. *Decomposition at 35° of rye straw extracted to remove hemicellulose and pectin.*

(Expressed on 100 g. dry extracted straw.)

Day	Dry matter	Organic matter	Total furfurald. yield	C. and B. cellulose	Furfurald. yield on C. and B. cellulose	Furfurald. from pentose groups in hemicellulose	Xylan associated with C. and B. cellulose	"Pure" cellulose by difference
—	100·00	98·52	5·73	90·01	5·01	0·72	7·77	82·24
8	89·02	87·54	3·60	77·84	2·86	0·74	4·46	74·38
16	82·71	81·23	3·22	69·40	2·60	0·62	4·06	65·34
24	76·49	75·01	2·68	63·25	2·13	0·55	3·34	59·91
32	73·99	72·51	2·44	59·24	2·06	0·38	3·22	56·02
48	68·85	67·37	2·20	52·30	1·62	0·58	2·53	49·77

The result of the preliminary extraction, admittedly a severe one, was to remove all but a trace of free hemicellulose, and to lower the content of lignin from 19·5 % to 9·3 %. At the same time the xylan associated with the Cross and Bevan cellulose product was also reduced considerably.

In spite of the absence of free hemicelluloses, the material decomposed quite rapidly, practically 40 % of the cellulose being removed in 7 weeks. It will be seen from Fig. 7 that the loss of cellulose coincides almost exactly with the loss of organic matter throughout, despite the fact that the xylan

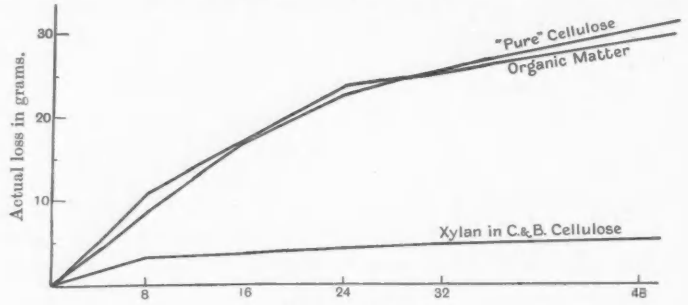

Fig. 7. Decomposition of extracted rye straw at 35°. (Expressed on 100 g. extracted straw.)

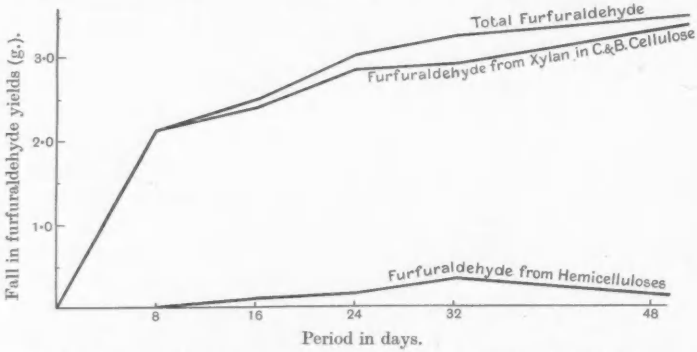

Fig. 8. Decomposition of extracted rye straw at 35°. Fall in furfuraldehyde yields. (Expressed on 100 g. extracted straw.)

associated with cellulose is also being removed. This gives additional evidence for the production from cellulose of some intermediate substance, for the apparent loss of cellulose and xylan at the end of 7 weeks is 37·5 g. expressed on 100 g. of extracted straw, while the total loss of organic matter over the same period is only 31 g. Fig. 8, which gives the fall in furfuraldehyde yields from the various groups, does not seem to indicate that there is any large production of transitional pentose or furfuraldehyde-yielding substances, although there is undoubtedly some.

To sum up, then, the decomposition seemed to take a perfectly normal course despite the absence of hemicelluloses. The organisms were able to obtain what energy they required from the cellulose, which was accordingly broken down rapidly. The decomposition was no doubt aided by the reduction of lignin, since a greater surface of cellulose would be exposed to attack. The final product, though light in colour, was not unlike well rotted manure.

Conclusions.

The biological decomposition of plant materials is the resultant of the activities of many organisms. Carbon compounds are required mainly as a source of energy for the organisms, though in part also for structural purposes. The most important carbonaceous substance in the tissue appears to be cellulose, and since it is rapidly removed under normal conditions it must be regarded as the chief source of energy for the micro-organisms. The hemicelluloses, of which there is usually a much lower percentage, are also utilised, and particularly so during the opening stages of the decomposition. Lignin has been shown by many workers to be an unavailable source of energy to ordinary micro-organisms. Being resistant it forms a barrier preventing them from reaching available sources of energy, and the ratio of available carbonaceous material to lignin must be some measure of the probability of decomposition of any material. The rapid early loss of hemicelluloses has probably two contributory causes. In the first place the hemicelluloses are presumably encrusting substances on the walls of the cell, which in any case would have to be removed in part before the cellulose could be attacked. Secondly, it is probably true to say that some hemicelluloses or some groups in the hemicellulose molecule are more easily available to the organisms than is cellulose. Together with the soluble components of the tissue, such as sugars and starches, they play an important part in the provision of easily obtainable energy, and in consequence promote the rapid growth and multiplication of the organisms in the early stages, thereby paving the way for the general attack on the cellulose. Only to the extent of hastening the incidence of the rapid breakdown of cellulose can the hemicelluloses be said to control the decomposition.

The somewhat obscure furfuraldehyde-yielding substance associated with the cellulose seems to play no very prominent part in this process. It appears to be decomposed but slowly and to a limited extent. The rate of its decomposition is probably determined by the rate of removal of the cellulose with which it is in such close association. It must, however, be regarded as an available and not a resistant constituent.

It seems therefore that the hemicelluloses must be deposed from the place of pre-eminent importance assigned to them by Rege [1927] in this type of decomposition, and must be viewed as of equal importance as other available carbonaceous sources of energy, save possibly in the opening stages.

Note on the prediction of the decomposability of any mature plant material.

The prediction of the decomposability of any plant material solely from the results of chemical analyses cannot be wholly satisfactory, in view of the differences in structure which cannot be taken into account in any formula. Nevertheless, despite this drawback, some rough prediction is of considerable value when considering the decomposition of lesser known plant materials. Particularly is it so in the case of the production of artificial manure (Adco) by the method of Richards and Hutchinson [1924]. The relationship which Rege [1927] indicated between "pentosans" and lignin has frequently been employed successfully to predict the decomposition of new materials for this purpose. Although, as has been shown earlier, the basis upon which his predictions are made is not entirely correct, their success is due to the inverse relationship between "pentosans" and lignin, which appears to hold in most plant materials.

Any alternative method need only be approximate but must be relatively simple to carry out. The following is suggested, with some reserve, as a more satisfactory basis for prediction. It involves only the analyses which were necessary for the Rege method. The determinations which are necessary are lignin, total furfuraldehyde yield, Cross and Bevan cellulose, and furfuraldehyde on Cross and Bevan cellulose product. The food or energy factor, as Rege described it, is obtained by adding to the Cross and Bevan cellulose a figure for free hemicelluloses. An approximation for this last may be obtained by multiplying the free furfuraldehyde by 2. This is purely arbitrary, but probably more satisfactory than the Kröber factor, which is too low. In any case the factor 2 errs on the low side for most hemicelluloses, so that underestimation is more probable than over-estimation. This combined figure for available material is then divided by the figure for resistant material, or

Table VII. *Table demonstrating the use of a ratio to predict the decomposability of mature plant materials.*

(Figures expressed as g. per 100 g. dry matter.)

Material	C. and B. cellulose [A]	Total furfurald. yield [E]	Furfurald. from C. and B. cellulose [D]	"Free" furfurald. [$E-D=B$]	Lignin [C]	Ratio: $\frac{A+2B}{C}$	Decomposability
Ext. rye straw	90·01	5·73	5·01	0·72	9·3	9·8	Rapid
Rice straw	45·88	14·5	5·2	9·3	10·31	6·3	"
Oat straw A	51·7	16·8	7·5	9·3	14·18	5·0	"
Wheat straw	52·1	18·3	8·9	9·4	14·57	4·9	"
Barley straw	49·4	16·6	6·6	10·0	16·16	4·3	"
Oat straw B	53·14	16·08	5·99	10·09	18·5	4·0	"
Rye straw	55·27	17·05	5·64	11·41	19·5	4·0	"
Rushes	51·3	16·3	7·4	8·9	17·71	3·9	Slow
Maize straw	43·0	15·2	6·2	9·0	20·34	3·0	"
Willow wood	49·46	13·7	3·8	9·9	24·70	2·8	Very slow
Poplar wood	66·3	12·1	6·4	5·7	28·44	2·7	"
Ash wood	44·64	13·9	2·6	11·3	26·01	2·6	"
Birch wood	44·53	15·2	1·6	13·6	28·27	2·5	"
White ash wood	53·4	13·1	5·4	7·7	28·38	2·4	"
Pine wood	57·4	5·2	3·4	1·8	26·65	2·3	*Nil*
Fir wood	43·44	6·7	1·7	5·0	29·17	1·8	"

lignin content, the quotient being a measure of the decomposability of the material. It is

$$\frac{[(\text{Cross and Bevan cellulose} + 2\ (\text{total furfuraldehyde} - \text{cellulosic furfuraldehyde})]}{\text{Lignin}}.$$

The figures for a number of plant materials are given in Table VII. A few have been calculated from Rege [1927] and a few of the woods from König and Becker [1919].

The last column headed "Decomposability" gives the result of actual trials except in the case of one or two woods. The connection between the observed decomposability and the ratio is clear. If the ratio is above 4 the material decomposes rapidly, and below 3 only very slowly. It must be remembered that this ratio is solely an index of probability of decomposition under optimum conditions, and bears no relationship to the suitability of the material for making artificial manure or to the quality of any product which might be obtained. The amount and availability of the organic matter decomposed is important in that it controls the immobilisation of nitrogen and other inorganic ions to a less extent, and so indirectly, the quality of the manure. The ratio given above, however, is not a measure of the former, which can only be obtained by trial.

Summary.

1. The decomposition of straw under optimum conditions by a mixed natural flora in the presence of sufficient available nitrogen is investigated. The course of the decomposition is followed by frequent and detailed analyses, particular attention being paid to the various types of furfuraldehyde-yielding substances.

2. The most prominent feature of the decomposition is the rapid loss of cellulose, which accounts for the major part of the loss of organic matter. The cellulose therefore forms the chief source of carbonaceous food or energy material for the organisms.

3. The hemicelluloses appear to decompose very rapidly in the first few days, and then to remain at much the same level while the cellulose is still decreasing. This is presumably due to the fact that as hemicelluloses are chemically non-homogeneous, their biological availability may vary very considerably, differing in this from unlignified cellulose. Two reasons may be advanced for the early loss of hemicellulose. Firstly, as they are encrusting substances on the cell wall, they must need to be removed, in part at least, before the cellulose can be attacked. In the second place, some hemicelluloses or some component groups of the hemicellulose molecule are easily available and these together with the soluble plant substances must play an important part in promoting the rapid multiplication of the organism.

4. The xylan associated with the Cross and Bevan cellulose fraction in this straw is not unavailable but is removed only slowly, the rate presumably being controlled by the loss of the cellulose with which it is in such intimate association.

5. There is definite evidence for the production of some intermediary substance which yields furfuraldehyde. That this is produced transitionally in the degradation of the cellulose is probable, though the support for this theory is scanty. The extent of the production of this substance is difficult to assess.

6. The loss of uronic acid from the straw is steady but small, and not paralleled by the apparent loss of hemicellulose. It seems likely that the uronic group is biologically less available, and that the residual hemicelluloses must contain intermediate degradation compounds of uronic content above normal.

7. The small amount of pectin present does not seem to be removed during normal decomposition, but only if acid conditions set in. There is some evidence that there is even a synthesis of pectin, or of some compound estimated as pectin.

8. Subject to structural and physical variations, it is agreed that the decomposition of plant materials in the presence of assimilable nitrogen is favoured by the food or energy factor, and retarded by the resistant factor. The food or energy factor must be regarded as being composed in mature materials of cellulose together with the available hemicelluloses, and the resistant factor as being the lignin fraction.

9. That the hemicelluloses are not of pre-eminent importance has been further supported by the decomposition of rye straw so extracted as to be practically hemicellulose-free.

10. A simple method for the prediction of probability of decomposition of any plant material is given, and its limitations outlined.

The writer wishes to express his indebtedness to Sir John Russell, Director of the Rothamsted Experimental Station, for placing at his disposal the facilities of the Station, and to the Department of Scientific and Industrial Research for a Senior Research Award, during the tenure of which this work was carried out. Especially are his thanks due to Mr E. H. Richards, Head of the Fermentation Department, whose ready advice and assistance throughout have been invaluable.

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CXLIX. THE INFLUENCE OF FEEDING EITHER FAT AND LIPASE OR LECITHIN ON THE SUGAR EXCRETION OF DEPANCREATISED DOGS.

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(*Received November 7th, 1929.*)

An extensive investigation of the metabolism of the phloridzinised dog has led Graham Lusk and co-workers to conclude, by analogy, that the metabolic disturbance in diabetes mellitus and in depancreatised dogs, due to the relative or complete lack of insulin, is a corresponding inability to oxidise carbohydrates. On the basis of this interpretation, these workers have strenuously opposed the view that sugar may be derived from fatty acid in the animal organism. The recent work of Wierzuchowski [1926], confirmed by Deuel, Wilson and Milhorat [1927], which demonstrated the oxidation of glucose by the phloridzinised dog, casts considerable doubt upon the above interpretation of the diabetic state.

The European school, whose views on this subject have been well reviewed by Geelmuyden [1923],—see also Von Noorden and Isaac [1927] and Macleod [1926]—has long advocated the over-production theory of diabetes which, in its simplest terms, attributes the glycosuria and hyperglycaemia of diabetes, not to a defect in oxidation of carbohydrates, but rather to an over-production of glucose from protein and fat, which causes its accumulation in spite of a continued ability to oxidise it. His recent work has led Macleod to advocate gluconeogenesis from fat, and to take that view of the diabetic disturbance which such a conclusion implies.

Macleod [1928] has recently summarised the evidence for the conversion of fat into carbohydrate in the animal organism. Since the publication of his monograph, the work here presented has been completed, and while the results are not of a crucial nature they lend further support to his conclusions. For this reason, and because they illustrate the physiological limitations of this type of experimentation, it seems desirable that these results be placed on record.

Methods.

Totally depancreatised dogs, maintained in an excellent state of health and nutrition by the use of insulin, were used. On the fifth day after withdrawal of all food and insulin, 50 g. of fat mixed with an equal amount of either a simple alkaline, pancreatic lipase extract or a castor bean suspension,

were administered by stomach tube. The lipase extracts, prepared according to Plimmer [1918], were given to facilitate the digestion and absorption of the fat from the intestine. Where lecithin was the substance administered, the addition of lipase was considered unnecessary, in view of the greater ease with which this substance undergoes hydrolysis [Bloor, 1926]. The fats used included olive oil, cotton-seed oil, butter and "intarvin" [Kahn, 1925].

Throughout the experiments, the sugar, nitrogen and total ketone excretion were determined for 24-hour periods. On the day of fat administration, the animal's R.Q. was determined in a carefully controlled respiratory cabinet of the Benedict type. In all cases a careful *post-mortem* examination was made for residual pancreatic tissue and if found the results were discarded for that animal. In some cases, determinations of the liver-glycogen were made by Pflüger's method. Urinary sugar, nitrogen and ketones were determined by the methods of Shaffer and Hartmann [1920–21], Kjeldahl, and Van Slyke [1917] respectively.

Results.

Owing to limitations of space, only one positive, one control and one negative experiment are reported in full (Table I). The remaining experiments are grouped in condensed tabular form (Tables II and III).

Discussion.

Exps. 1, 5 (Table II) and 6 (Table I) clearly show an excess sugar excretion, even after maximal deductions are made for the glucose which might have come from the glycerol portion of the fat, and from extra nitrogen excreted on the day of fat administration. That any of the extra glucose excreted could have come from the muscle-glycogen stores is unlikely. There is at least no evidence that the administration of a food material like a neutral fat can cause a displacement of glucose from the tissue stores and it can be seen from the tables that the control animals in the present experiments showed no such displacement. Since the minuteness of the liver-glycogen stores on the fifth day after withdrawal of food and insulin [Chaikoff, 1927] renders this organ insignificant as a source of the extra sugar excretion, it can be concluded that none of the extra glucose excreted in the positive experiments was derived from the carbohydrate stores of the animal. A comparison of Exps. 1, 5 and 6 with the control Exps. 7, 8 and 9, therefore, indicates that the extra sugar excreted must have been derived from fatty acid.

A large number of experiments yielded negative results (Table III) and there is no reason to doubt that, were the experiments carried on indefinitely, positive results would be obtained in about the same ratio to negative ones as in the present series. A brief consideration, however, of the limitations of this type of experiment, makes it apparent that, with our direct method of approach:

(1) it is quite unlikely that results more pronouncedly positive could ever be obtained;

(2) the number of negative results might reasonably be expected to be relatively great;

(3) to such positive results as have been obtained must be conceded an added significance.

Table I. *Details of typical experiments.*

Day of exp.	Remarks	Total ketone excretion g.	Sugar output g.	N output g.	D : N	
I. *Showing sugar formation from fat. Exp. No. 6.*						
1	Stopped food and insulin.					
2	Weight 7·73 kg.	0	87·08	4·27	20·39	—
3	" 7·56 "	0·83	49·41	4·95	9·98	—
4	" 7·22 "	3·87	30·72	4·95	6·21	—
5	25 g. lecithin *per os.* 15 g. lecithin subcutaneously. Food and insulin failed to prevent death on following day. Weight 7·02 kg.	4·86	40·11	5·35	7·50	R.Q. 0·693. Calories 54·6 per kg. *per diem.* *Sugar from glycerol 2·65 g. †Sugar from protein 1·46 g. Sugar from fatty acid 5·28 g.
II. *Control with mineral oil. Exp. 9.*						
1	Stopped food and insulin.					
2	Weight 7·05 kg.	0	43·60	5·01	8·70	—
3	" 6·70 "	0·24	23·10	4·48	5·16	—
4	" 6·48 "	1·34	23·43	5·59	4·19	—
5	55 cc. mineral oil + 55 cc. pancreatic lipase. Promptly recovered on food and insulin. Weight 6·25 kg.	1·93	21·70	5·66	3·83	R.Q. 0·700. Calories 79·2 per kg. *per diem.* *Sugar from glycerol 3·76 g. †Sugar from protein 0·26 g. Sugar from fatty acid 0 g.
III. *No sugar formation from fat. Exp. 4.*						
1	Stopped food and insulin.					
2	Weight 8·1 kg.	Trace	85·55	5·28	16·21	—
3	" 7·8 "	1·50	39·05	4·56	8·57	—
4	" 7·6 "	4·27	21·10	5·27	4·00	—
5	50 g. cottonseed oil + 55 cc. pancreatic lipase. Recovered on food and insulin. Weight 7·3 kg.	7·39	33·17	7·38	4·49	R.Q. 0·696. Calories 71·7 per kg. *per diem.* *Sugar from glycerol 3·59 g. †Sugar from protein 7·70 g. Sugar from fatty acid 0·78 g.

* *Calculation for sugar from glycerol.*

Animal required 54·6 calories per kg. *per diem*; animal weighed 7·02 kg.; therefore animal required $7·02 \times 54·6 = 383·3$ calories *per diem.*

Nitrogen output = 5·35 g.; therefore $5·35 \times 6·25 = 33·44$ g. protein were utilised; and therefore $33·44 \times 4·1 = 137·1$ calories were derived from protein.

Assuming that no energy was derived from carbohydrate, $383·3 - 137·1 = 246·2$ calories were derived from fat. Therefore $\frac{246·2}{9·3} = 26·5$ g. fat were utilised for energy; and therefore $\frac{26·5}{10} = 2·65$ g. glycerol is maximum amount available for conversion into glucose.

† *Calculation for sugar from protein.*

Nitrogen output on 5th day of experiment = 5·35 g.
" 4th " = 4·95 g.
Extra nitrogen output on 5th day = 0·40 g.
Glucose available from extra nitrogen = $0·40 \times 3·65 = 1·46$ g.

It will be noted that liver-glycogen was increased in Exps. 10 and 13 but, although this offsets to some extent the lack of extra sugar excretion, there still remain ten experiments which are quite negative in their results. In order that an experiment of the type here reported could yield a positive result, it would be necessary for some of the administered fat to be carried

Table II. *Condensed results of experiments showing sugar formation from fat, and of two control experiments.*

Exp. No. and wt. of dog	Administered *per os* on 5th day of exp.	Total ketone excretion g.	*Extra sugar output g.	*Extra N output g.	D : N	
†1 8·38 kg.	50 g. olive oil + 50 cc. castor bean suspension	0·31	9·20	0	6·76	R.Q. 0·702. Calories 60 per kg. *per diem*. Sugar from glycerol 4·35 g. Sugar from fatty acid 4·85 g.
†5 6·47 kg.	30 g. lecithin	2·33	11·07	0	7·78	R.Q. 0·708. Calories 77 per kg. *per diem*. Sugar from glycerol 4·16 g. Sugar from fatty acid 6·91 g.
†7 4·60 kg.	55 cc. mineral oil + 55 cc. water	0·14	2·17	0·53	3·02	R.Q. 0·696. Calories 72 per kg. *per diem*. Sugar from glycerol 2·30 g. Sugar from protein 1·93 g.
†8 6·19 kg.	55 cc. mineral oil + 55 cc. water	0·59	0	0·39	3·29	R.Q. 0·719. Calories 91 per kg. *per diem*. Sugar from glycerol 4·48 g. Sugar from protein 1·42 g.

* Over the preceding 24 hours. † (1) Died; (5) died; (7) recovered; (8) recovered.

Table III. *Condensed results of experiments not showing sugar formation from fat.*

Exp. No. and wt. of dog	Administered *per os* on 5th day of exp.	Total ketone excretion g.	Extra sugar output g.	Extra N output g.	D : N	
2 6·76 kg.	50 g. olive oil + 55 cc. pancreatic lipase	—	0	—	—	R.Q. 0·704. Calories 86 per kg. *per diem*.
3 6·40 kg.	50 g. cottonseed oil + 55 cc. castor bean suspension	—	2·45	—	—	R.Q. 0·702. Calories 62 per kg. *per diem*.
*10 5·80 kg.	50 g. cottonseed oil + 55 cc. pancreatic lipase (on 6th day of fasting)	1·40	0	0	2·78	R.Q. 0·692. Calories 73 per kg. *per diem*. Sugar from glycerol 3·22 g.
11 8·10 kg.	50 g. cottonseed oil + 55 cc. pancreatic lipase (on 6th day of fasting)	1·00	1·93	0·55	2·68	R.Q. 0·686. Calories 57 per kg. *per diem*. Sugar from glycerol 3·81 g. Sugar from protein 2·00 g.
12 7·61 kg.	50 g. olive oil + 55 cc. pancreatic lipase (on 5th and 6th days of fasting)	0·53	7·40	2·04	2·99	R.Q. 0·701. Calories 54 per kg. *per diem*. Sugar from glycerol 3·2 g. Sugar from protein 7·45 g.
*13 9·50 kg.	Ran parallel to Exp. 12. Male dog. Did not catheterise.					
14 6·48 kg.	50 g. butter-fat + 55 cc. pancreatic lipase	0·19	2·86	0·41	2·74	R.Q. 0·701. Calories 73 per kg. *per diem*. Sugar from glycerol 3·74 g. Sugar from protein 1·50 g.
15	Died on 5th day of exp.					
16 8·40 kg.	50 g. olive oil + 55 cc. pancreatic lipase	4·96	0	0	2·99	R.Q. 0·702. Calories 73 per kg. *per diem*. Sugar from glycerol 4·58 g.
17 5·00 kg.	50 g. olive oil + 55 cc. pancreatic lipase	4·58	0·46	0·61	2·73	R.Q. 0·709. Calories 77 per kg. *per diem*. Sugar from glycerol 2·55 g. Sugar from protein 2·23 g.
18 7·50 kg.	50 g. "intarvin" + 55 cc. pancreatic lipase	4·66	6·62	1·09	4·71	R.Q. 0·654. Calories 79 per kg. *per diem*. Sugar from glycerol 4·37 g. Sugar from protein 3·98 g.

* (10) 3·39 g. extra glycogen in liver. (13) 13·77 g. extra glycogen in liver.

Calculation for extra glycogen in the liver.

Highest average value for liver-glycogen in diabetic dogs on the 5th day after the withdrawal of food and insulin is 0·19 % [Chaikoff, 1927].

Assuming the liver weighs 5 % of total body weight $= \frac{5}{100} \times 5800 = 290$ g.

Estimated glycogen in the liver $= \frac{0{\cdot}2}{100} \times 290 = 0{\cdot}58$ g.

Actual glycogen in the liver $= \frac{1{\cdot}37}{100} \times 290 = 3{\cdot}97$ g.

Extra glycogen in the liver $= 3{\cdot}97 - 0{\cdot}58 = 3{\cdot}39$ g.

to the liver, and there converted into glucose, and this process would have to be superadded on the pre-existing level of gluconeogenesis from tissue protein and fat. But the supply of fat does not necessarily determine its utilisation by the liver. As Macleod has suggested (personal communication):

> "The factor upon which gluconeogenesis from fat will depend is the call of the liver for fat rather than the supply which may be carried to this organ by the blood as a result of fat absorption. If this view be correct, then it might quite well happen in diabetes that in some animals the liver would not yet have developed its demand for fat at the time when the fat-feeding was taking place, in which case, of course, there would be no evidence of gluconeogenesis. In other words, the conditions would be similar to those existing in very fat animals, with plenty of 'depot' fat, some of which, as we know, develop the gluconeogenesis at an earlier stage than others."

This view is borne out by the fact that in most of the above experiments—positive, negative and control—the calculated utilisation of fat is of the same order of magnitude. If the positive results depend on the coincidence of the fat administration with a somewhat increased capacity of the liver for gluconeogenesis, it is hardly surprising that a large number of negative results should be obtained.

The low respiratory quotient obtained in Exp. 18 is very interesting in view of the support it lends to the conversion of fat to carbohydrate. The fact, however, that in most of the experiments the R.Q. does not fall significantly below 0·7, is apparently inconsistent with such a process which, by causing a retention of oxygen, should bring about a lower R.Q. This view rests upon the assumption, not supported by recent work, that the diabetic animal does not burn carbohydrate. If we do not make this assumption, then the more or less constant diabetic R.Q. can be readily explained as being the algebraic sum of two quotients—an R.Q. of 1·0 due to the oxidation of carbohydrates, and an R.Q. of 0·2 resulting from gluconeogenesis from fat [Macleod, 1928, p. 78].

It will be observed that, in the clearly positive experiments, the animal died in every case, despite all efforts to revive it. This further illustrates the physiological limitations which must be overstepped in order to obtain a positive result. In a totally diabetic animal, which has been deprived of all food and insulin for four days, and is already very ill as a result of the high level of gluconeogenesis, we attempt to drive this process to its utmost extreme. When we succeed in any measure, the animal dies. There is, therefore, only a narrow margin within which we can get the animal which does show a positive result to survive long enough to complete the experiment.

Finally, it may be noted in passing that the administration of "intarvin" did not lower the ketone excretion in Exp. 18. On the day of "intarvin" administration the total ketone excretion increased from 2·445 g. of the preceding 24 hours, to 4·659 g. This does not agree with the work of Kahn [1925], but is in accord with the recent work of Moore *et al.* [1928].

SUMMARY.

1. In three totally depancreatised dogs, the administration of fat was followed by an excretion of extra glucose which could not be accounted for by the glycerol portion of the fat, the nitrogen excretion and the carbohydrate stores of the animal.

2. Gluconeogenesis from fatty acid is therefore confirmed.

3. The physiological limitations of this type of experimentation are pointed out, as accounting for the many negative experiments obtained.

4. In one experiment, where "intarvin" was the fat administered, it failed to show its supposed antiketogenic action.

It is a pleasure to acknowledge the advice and direction of Prof. J. J. R. Macleod and, since his departure, the kind interest of Prof. C. H. Best.

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CL. *POST-MORTEM* FORMATION OF METHAEMOGLOBIN IN RED MUSCLE.

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(*Received September 24th, 1929.*)

THE colour of a red muscle is due to haemoglobin present (*a*) as muscle-haemoglobin and (*b*) in any blood corpuscles remaining in the capillaries. The amount of muscle-haemoglobin appears to be independent of the degree of blood removal and dependent on the type of muscle and the age and condition of the animal [Whipple, 1926]. In lean meat, the tissue used in the present paper, it is probable that muscle-haemoglobin is a large fraction of the total pigment [cf. Whipple, 1926, Table IV].

It has long been known that methaemoglobin is slowly formed on exposure of haemoglobin solutions to oxygen. If muscle has any oxidising mechanism which functions in the absence of oxygen no evidence has been obtained that it is responsible for *post-mortem* methaemoglobin formation[1]. It is necessary therefore to consider the conditions governing the penetration of oxygen into the tissue.

The presence of oxygen in the tissue.

The tissue used has been the red flexor muscles of the fore leg of the ox. They were removed from the limb 18 to 24 hours after death and unless otherwise stated kept in a room maintained at 0°.

It was found that this tissue had a small oxygen uptake. Therefore, given sufficient time, a "steady state" must be reached where the depth of oxygen penetration is determined by the rate of diffusion of oxygen into the tissue and the oxygen consumption. With large pieces of tissue the surface can be considered as a plane. This case has been treated by Warburg [1923] and Hill [1928, 2] who have shown that the greatest depth, d cm., to which oxygen penetrates is given by the equation

$$d = \sqrt{\frac{2c_0 D}{A}} \qquad \text{.....(1),}$$

where c_0 = oxygen pressure in atmospheres at the surface of the tissue.

D = diffusion coefficient of O_2 through the tissue expressed in cc. O_2 (0°, 760 mm. Hg) diffusing in 1 min. across 1 cm.^{2} when pressure gradient of O_2 is 1 atmosphere per cm.

A = oxygen uptake of the tissue expressed in cc. O_2 (0°, 760 mm.) consumed by 1 cc. of tissue in 1 min.

[1] No detectable amounts of methaemoglobin were formed when the tissue was stored in pure nitrogen.

A slice of the tissue several cm. square and slightly more than 1·5 mm. thick was cut freehand with a razor and laid between two sheets of plate glass. Two glass strips 1·5 mm. thick were placed between the plates, and the tissue was carefully compressed to this thickness so that oxygen could diffuse only across the edges of the slab (Fig. 1, p. 1395). This arrangement allowed spectroscopic examination of the tissue pigments *in situ* at different distances from the air-tissue interface, using a Zeiss microspectroscope with "pointolite" illumination. The oxygen uptake of the tissue can be demonstrated by placing between the plates a slice previously exposed to air for a short time. It will be found, except in a sharply defined outer red zone, that the oxyhaemoglobin is reduced to the purplish haemoglobin because of oxygen removal by the tissue.

A rough value of d can be obtained from the width of the outer zone. It can be safely assumed that where the characteristic purple colour appears the ratio $\frac{\text{oxyhaemoglobin}}{\text{reduced haemoglobin}}$ is less than unity. If the oxygen dissociation curve of muscle-haemoglobin is similar to that of haemoglobin in solution this ratio is unity at 0° when the partial pressure of oxygen is small; let it be taken as 4 mm. Hg [cf. Brown and Hill, 1923]. The partial pressure, c atmospheres, of oxygen in an element dz of tissue, distance z cm. from the surface, is given by the equation (Warburg)

$$c = \frac{Az^2}{2D} - \frac{Azd}{D} + c_0$$

substituting

$$d^2 = \frac{2c_0D}{A}$$

$$c = c_0\left(\frac{z}{d} - 1\right)^2 \qquad \text{.....(2),}$$

or

$$\beta^{\frac{1}{2}} = 1 - \alpha \qquad \text{.....(3),}$$

where β is the fraction (c/c_0) of the surface pressure of oxygen in the element dz and α is the ratio (z/d) of the distance from the surface of this element to the total distance of oxygen penetration. This ratio for the element where the O_2 pressure is 4 mm. is α where

$$\alpha = 1 - \left(\frac{4}{150}\right)^{\frac{1}{2}} = 0{\cdot}84.$$

Hence when a steady state is reached the width of the red outer strip is at least 84 % of the total distance of oxygen penetration. For the present purpose a measurement of this width (hereafter denoted d) gives a fair approximation to d. A hand lens and a scale graduated in 0·5 mm. were used and 12 readings were taken on each tissue slice; the mean is probably correct to approximately 10 %.

The width of the red strip reaches a definite value in a few hours at 0° and thereafter only increases slowly. The slow increase is not due to a non-attainment of a "steady state," for the same values of d were obtained (after less than 7 hours) with two halves of a tissue slice, one of which was exposed to oxygen and the other to nitrogen for 2 hours before being placed between the

glass plates. In this case the values were approached from two sides. The increase seems to indicate (cf. equation (1)) a decrease in the rate of oxygen consumption slow in comparison with the rate of diffusion of oxygen.

The effect of previous freezing in air on the depth of oxygen penetration at 0° was determined. The slice was cut from the interior of the frozen muscle and immediately thawed by contact with two glass plates at room temperature. The details of one experiment are given in Table I[1]. The control slice was cut from a portion of the same muscle which had been kept at 0°.

Table I.

Temperature 0°. Thawed tissue previously frozen for 18 hours at −10°.

	d cm.	
Hours	Control tissue	Thawed tissue
14	0·24	0·20
40	0·29	0·25
84	0·37	0·37
111	0·39	0·42
135	0·45	0·52

Similar results were usually obtained, d increased slowly at an approximately linear rate and the rates for thawed and untreated muscle were similar. In Table II $k_1 = \left(\frac{d.d}{dt}\right)$ is the linear rate of increase in cm./hr. and d_1 is the value of d obtained by extrapolating to zero time (time of thawing). The values are the mean of all the results obtained[2] (using beef from 16 animals).

Table II.

Temperature 0°.

	d_1	k_1
Control tissue	0·21	0·0019
Thawed tissue	0·19	0·0027

Different temperatures of freezing have been used (from −3° to −20° and also in liquid air) and the duration of time in the frozen state has been varied (up to 3 weeks) but no marked effects were observed.

Increase of temperature decreases the value of d_1; this agrees with the usual low temperature coefficient of diffusion as compared with that of a process such as oxygen uptake. Mean values of d_1 at three temperatures were 0°, 0·22 cm.; 5°, 0·16 cm.; 15°, 0·09 cm. (increase in d was followed only for a short period at the two higher temperatures).

It is of interest to calculate from equation (1) the rate of oxygen uptake at 0° using the mean value of d_1 from Table II. The value obtained,

$$A = 0{\cdot}8 \times 10^{-4}$$

[1] The appearance in some cases of a brown discoloration in the oxygen region due to methaemoglobin formation is described later.

[2] There was a small difference in the behaviour of the different flexor muscles. With the more loosely textured, less pigmented muscle in the deeper part of the foreleg, k_1 was larger than the values in Table II.

is necessarily only approximate[1]. After 100 hours' standing at 0° A has fallen to $0{\cdot}2 \times 10^{-4}$. Taking the mean value found by Hill [1928, 1] for the respiration of resting frog's muscle at 20°, $A = 7{\cdot}8 \times 10^{-4}$, and assuming a temperature coefficient of 2·5 per 10°, for living muscle $A = 1{\cdot}2 \times 10^{-4}$ at 0°. It is possible that the oxygen consumption in the present case may be due partly to the oxidation of unsaturated constituents of injured cells.

Hill [1928, 2] has considered the case of a thick, isolated, living muscle where the lactic acid formed anaerobically diffuses outward and is destroyed in the oxygen region. In the steady state the depth to which oxygen penetrates should diminish rapidly as the thickness of the muscle increases. With the tissue used in the present work the values of d were independent of the area of the tissue slice (which corresponds to the thickness of the muscle). This suggests either that with this tissue there is no interaction between oxygen and the products of anaerobic activity or more probably that there is little or no anaerobic production of lactic acid.

Formation of methaemoglobin in the tissue.

From the above results it can be concluded that methaemoglobin formation in muscle exposed to air should be confined to a thin surface layer and that freezing and thawing of the tissue in air[2] has no significant influence on the depth of the layer.

Conant [1923, 1924, 1926, 1928] has shown that methaemoglobin and reduced haemoglobin form a reversible oxidation-reduction system of the ferri-ferrous type. Oxyhaemoglobin is therefore not an intermediate step in the formation of methaemoglobin. Conant suggested the following equation for the oxidation by oxygen

$$\underset{\text{Reduced haemoglobin}}{2H_2X} + O_2 \rightarrow H_2O_2 + \underset{\text{Methaemoglobin}}{2HX}$$

Neill [1925] has pointed out that increase of the oxygen pressure decreases the concentration of the other reactant by the formation of oxyhaemoglobin. There should therefore be an optimum oxygen pressure. Neill and Hastings [1925], using sterile haemoglobin solutions, alone and in the presence of various catalysts, found that the rate of reaction was greatest at an oxygen pressure corresponding approximately to that required for 50 % combination of haemoglobin with oxygen, *i.e.* to a small oxygen pressure.

[1] The value of d_1, 0·20 cm., was divided by the factor 0·84 to obtain a maximum value for the depth of oxygen penetration, *i.e.* a minimum value for the oxygen uptake. The diffusion coefficient of oxygen through muscle at different temperatures has been determined by Krogh 1918–1919]. The calculated value of A depends of course on the applicability of these values to the tissue used.

[2] It was found that after freezing in saturated NaCl solution the value of d for the thawed tissue was greatly increased. Apart therefore from the effect of NaCl on methaemoglobin formation the increased accessibility of the muscle pigment to oxygen leads to the formation of a much greater quantity of methaemoglobin.

If a similar dependence on the pressure is assumed for the action of oxygen on muscle haemoglobin it would be expected that the rate of methaemoglobin formation in the oxygen region would be greatest in an element at some distance from the surface. This has been found to be the case.

Methaemoglobin in sufficient amounts can be detected spectroscopically in the presence of the other substances whose spectra are observed on examination of muscle (oxy- and reduced haemoglobin and cytochrome [Keilin, 1925]). Tissue slices were cut from the interior of the muscle as the oxygen region of the original muscle if not used immediately may contain a certain amount of methaemoglobin and may have been altered by factors such as drying, etc. It was also necessary to prevent drying of the edges of the slices during storage. These slices enclosed between two glass plates were stored at 0° and examined spectroscopically at intervals. Methaemoglobin was formed only in the oxygen region and more rapidly in the parts further from the air-tissue surface. The difference in rate at different distances from this surface was great enough to form, after a certain time, a yellow-brown zone (containing mainly methaemoglobin) apparently immediately adjoining the region of reduced haemoglobin, while nearer this surface the tissue was still reddish (Fig. 1).

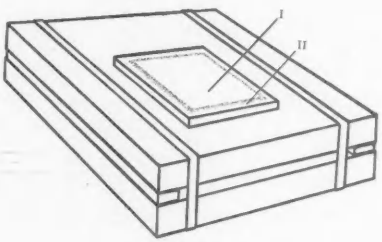

Fig. 1.

I. Region of reduced haemoglobin.
II. Oxygen region containing brown zone due to methaemoglobin formation.

The width of the brown zone increased with time at a somewhat greater rate than the width of the oxygen region d. On the side further from the surface it increased at the same rate as d, since the band always adjoined the reduced haemoglobin. On the side nearer the surface it spread more slowly and after a certain time became indistinct as the amount of methaemoglobin in the layers nearer the surface increased.

The brown discoloration was usually detectable sooner in the thawed tissue than in the control tissue. In some cases the brown layer d_B was sufficiently well defined to be roughly measurable. Table III contains the details of one of the better experiments, d_R is the width of the reddish layer nearer the surface. In other cases these measurements were not possible and the approximate nature of those quoted must be emphasised.

Table III.

Temperature 0°.

Tissue	Hours	d	d_R	d_B
Thawed	20	0·26	0·21	0·05
	53	0·35	0·22	0·13
	69	0·38	0·19	0·19
	98	0·46	*ca.* 0·13	*ca.* 0·33
	141	0·57	Indefinite	Indefinite
Control	20	0·27	0·27	—
	53	0·32	0·32	—
	69	0·37	< 0·37	—
	98	0·41	0·29	0·12

The results can be expressed in terms of the time required for the appearance of a marked colour change. It is assumed, as is the case, that d_B increases initially at approximately the same rate as d and the maximum value of t when $d_B = 0$ is calculated. Table IV contains these values for several experiments where the thawed tissue had been frozen at $-10°$ for approximately 30 hours; No. 3 is the experiment contained in Table III.

Table IV.

Temperature 0°. t (hours).

No.	1	2	3	4	5	6	7	Mean
Control tissue	40	60	20	50	90	80	100	60
Thawed tissue	30	10	0	30	60	40	50	30

Different temperatures of freezing have been used and the impression was gained that the ratio t thawed/t control was nearer unity after freezing at higher temperatures (*e.g.* $-3°$) than at lower temperatures (*e.g.* $-20°$).

The values in Table IV are little more than qualitative but it seems permissible to conclude that freezing at $-10°$ and thawing the tissue increases the subsequent rate of methaemoglobin formation.

The relation of oxygen pressure to the rate of formation of methaemoglobin.

An attempt has been made to consider in more detail the relation of oxygen pressure to the rate of formation of methaemoglobin in tissue.

The equilibrium between oxygen and haemoglobin has been expressed by equations (4) and (5). It is now known that this treatment is too simple but, as the equations hold over a fair range of oxygen pressures and the question is still undecided, it seems justifiable to use them in an approximate treatment.

$$\underset{\text{reduced haemoglobin}}{(H_3X)_n} + nO_2 \rightleftharpoons \underset{\text{oxyhaemoglobin}}{(H_3X.O_2)_n} \qquad \text{.....(4),}$$

$$\frac{[(H_3X)_n] \cdot p^n}{[(H_3X.O_2)_n]} = K \qquad \text{.....(5).}$$

The oxygen concentration and hence the equilibrium constant is expressed in terms of the partial oxygen pressure.

The concentration of reduced haemoglobin at time t

$$= [a - x] . \frac{K}{K + p^n}$$

where $[a - x]$ is the total concentration of haemoglobin (oxy- and reduced) at time t. When the oxidation of reduced haemoglobin is represented by the equation

$$2\,(H_3X)_n + nO_2 \rightarrow nH_2O_2 + 2\,(H_2X)_n$$

the rate of methaemoglobin formation dx/dt is

$$\frac{dx}{dt} = k\,[(H_3X)_n]^2 . p^n$$

$$= k\,[a - x]^2 . \frac{K}{(K + p^n)^2} . p^n .$$

At a constant pressure of oxygen

$$\frac{dx}{dt} = k'\,[a - x]^2 .$$

The optimum pressure of oxygen is obtained by differentiating k' with respect to p and equating to zero

$$\frac{dk'}{dp} = \frac{kK^2 n p^{n-1}}{(K + p^n)^3} . (K - p^n) = 0$$

$$p_{opt} = K^{1/n}$$

i.e. the optimum oxygen pressure is such that the concentration of reduced haemoglobin equals the concentration of oxyhaemoglobin[1], as found by Neill and Hastings [1925].

As the oxygen dissociation curve of muscle-haemoglobin is unknown the data of Brown and Hill [1923] on human blood have been taken and the distance from the surface of the tissue element in which the optimum pressure exists has been calculated from equation (3)[2]. Table V contains these distances in terms of the fractional distance $\alpha\ (=z/d)$. Thus at 0° if the depth of the oxygen region is 1 cm. and the optimum oxygen pressure is 4·0 mm. Hg methaemoglobin should be most rapidly formed at a depth of 0·84 cm.

Table V.

Temperature	p opt.	α opt.
0°	4·0 mm.	0·84 cm.
10	6·4	0·79
20	11·4	0·72

The relative times required for the transformation of a given fraction of the total pigment at different depths, assuming the reaction suggested by Conant, have been calculated. This should give at least a qualitative idea of

[1] Lower pressures are obtained if other reactions are assumed for the oxidation to methaemoglobin.

[2] The influence of the change in the depth of the oxygen region with time is considered below.

the course to be expected in tissue. In a given element the oxygen concentration is constant[1] and the rate of methaemoglobin formation may be written

$$\frac{dx}{dt} = k'[a - x]^2.$$

The time $t_{0\cdot5}$ taken for transformation of half the total haemoglobin (oxy- and reduced) is obtained by integration and substitution of

$$x = 0\cdot5a,$$

$$t_{0\cdot5} = \frac{1}{k'a}.$$

In any element

$$t_{0\cdot5} = \frac{(K+p^n)^2}{akK^2p^n}.$$

Substituting different values of p corresponding to different fractional depths a the relative times at 0° in Table VI were obtained, the half-time in the optimum element being taken as unity. Two values of n have been used, 1·0 and for comparison 2·2 [cf. Brown and Hill, 1923].

Table VI.

		Relative times for 50 % transformation	
a (cm.)	p mm. Hg	$n=1$	$n=2\cdot2$
0·95	0·38	3·2	45
0·90	1·5	1·3	3
0·84	4	1·0	1
0·68	15	1·5	5
0·50	38	2·9	36
0·29	75	5·2	159
0	150	9·9	726

In both cases there is a sufficient decrease in rate as the distance from the optimum element increases to account for the observed differences.

It has so far been assumed that the oxygen pressure in any element remains constant, *i.e.* the depth of the oxygen region is constant. Since d increases linearly the optimum element moves at a constant rate away from the surface. Increase in d increases the oxygen pressure at any point and increase in p in an optimum element decreases the rate of methaemoglobin formation. It can be seen that the smaller the rate of increase of p the slower is the decrease in the rate of methaemoglobin formation.

It can be shown for an optimum element that

$$\frac{dp}{dt} = \frac{\text{constant}}{d}.$$

Thus the partial pressure of oxygen, c atmospheres, at any point depth z in the oxygen region is given by equation (2)

$$c = c_0\left(\frac{z}{d} - 1\right)^2 \qquad \text{.....(2).}$$

[1] The amount of oxygen consumed in the pigment change is negligible in comparison with the rate of diffusion of oxygen since both the amount of pigment and the rate of change are small.

Substituting $d = d_1 + k_1 t$ and differentiating

$$\frac{dc}{dt} = -\frac{2c_0 k_1 z (z - d_1 - k_1 t)}{(d_1 + k_1 t)^3}$$

$$= \frac{2c_0 k_1 z (d - z)}{d^3}.$$

Considering an optimum element $z = \alpha_{\text{opt.}} d$

$$\frac{dp}{dt} = 760 . \frac{dc}{dt} = \frac{1520 . c_0 . k_1 . d^2 . \alpha_{\text{opt.}} (1 - \alpha_{\text{opt.}})}{d^3}$$

$$= \frac{\text{constant}}{d}.$$

Hence it follows that in succeeding optimum elements the rate of increase of p is smaller since d has increased. The mean rate of methaemoglobin formation is therefore more nearly maintained at the optimum rate as d increases. At a certain value of d the mean rate may be maintained at a high value for a sufficient time to produce rapidly in a given element (and hence in all succeeding elements) the colour change from red to brown. This appears to explain the increase in width of the brown zone on the side further from the tissue surface at the same rate as the increase in width of the oxygen region.

The general correctness of the suggested relation between oxygen pressure, depth of the oxygen region and methaemoglobin formation is supported by the following experiment. A large muscle was frozen at $-10°$ and placed in cylinder nitrogen at 0°. After 40 hours the surface of the muscle was brown. On cutting a transverse section it was found that a thin brown layer (less than 1 mm. thick) containing mainly methaemoglobin covered the surface; beneath this layer the pigment was in the form of reduced haemoglobin. As cylinder nitrogen contains about 1 % of oxygen the depth of the oxygen region should be approximately four times smaller than in air (cf. equation 1) and as the oxygen pressure in the region (ranging from approximately 8 mm. to zero) lies near to the optimum pressure the rapid formation of a thin brown surface layer can be explained. The surface of a similar muscle after freezing and standing in air for 40 hours at 0° was red.

Summary.

1. Some of the factors concerned in the formation of methaemoglobin in pigmented muscles have been considered.

2. As the formation of methaemoglobin in the tissue used was not observed in the absence of oxygen, the depth of penetration of oxygen into the tissue was investigated. After standing a few hours at 0° a "steady state" was established such that the depth of oxygen penetration was approximately 2 mm.; after 100 hours the depth had increased linearly to approximately 4 mm. The increase in depth was attributed to a slow decrease in the oxygen consumption of the tissue. Freezing and thawing the tissue had no significant effect.

3. Methaemoglobin was slowly formed in the oxygen region of tissue exposed to air and most rapidly at some distance from the tissue-air interface.

Freezing and thawing appeared to increase the rate of methaemoglobin formation.

4. The dependence of the rate on the position in the oxygen region was in qualitative agreement with the expected behaviour.

I wish to thank Dr D. Keilin, F.R.S., for his continued interest and help.

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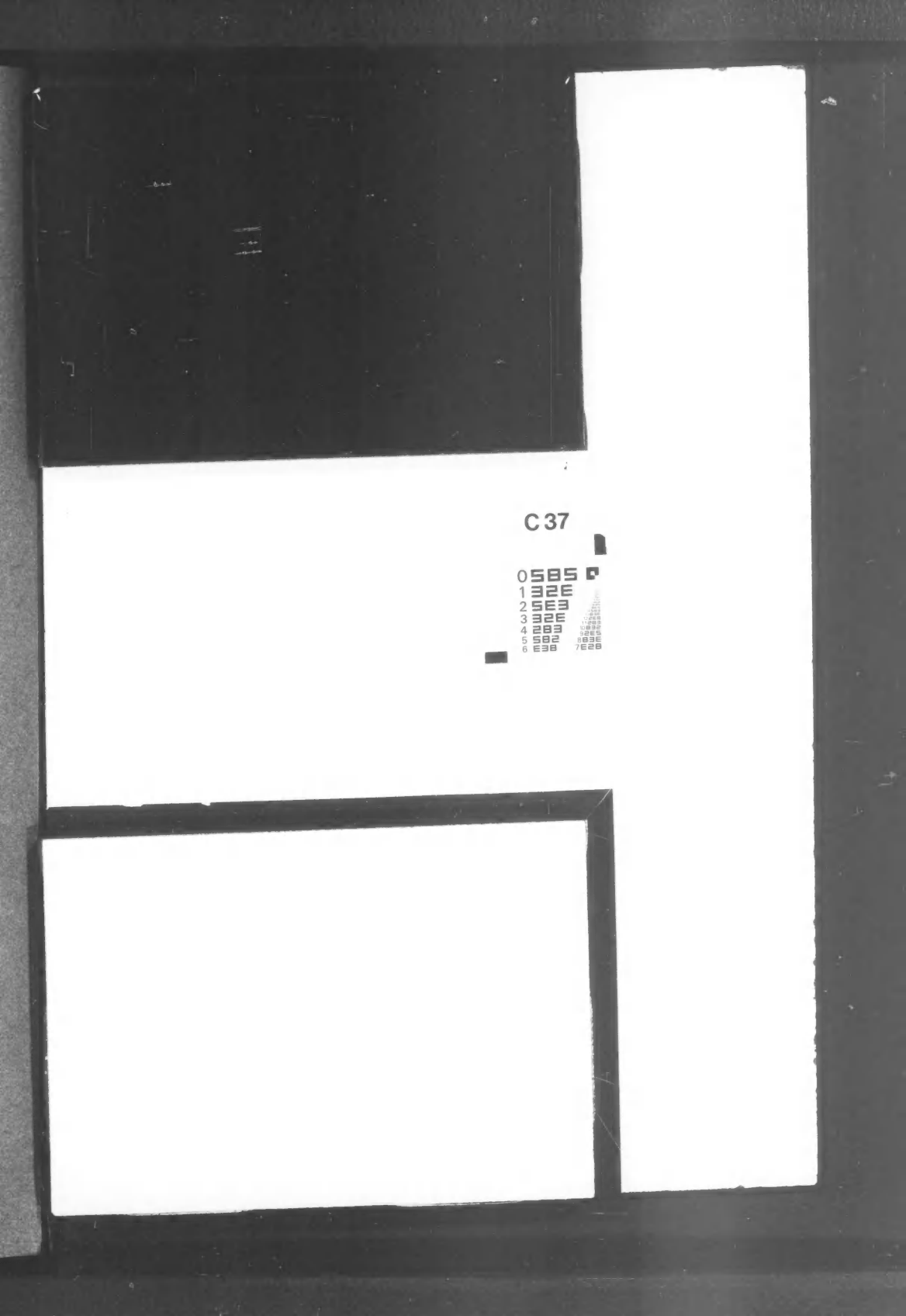
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